

1990

Qualitative characterization of the endogenous cytokinins in red pine and aspects of their influence on seedling development

Richard Meilan
Iowa State University

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pine and aspects of their influence on seedling development**

Meilan, Richard, Ph.D.

Iowa State University, 1990

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**300 N. Zeeb Rd.
Ann Arbor, MI 48106**

**Qualitative characterization of the endogenous cytokinins in
red pine and aspects of their influence on seedling development**

by

Richard Meilan

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

**Departments: Forestry
Botany**

**Majors: Forestry (Biology-Wood Science)
Botany (Physiology & Molecular Biology)**

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For the Major Departments

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For the Graduate College

**Iowa State University
Ames, Iowa
1990**

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(B) 1.0 μ M BAP + 0.1 μ M NAA

(C) 1.0 μ M BAP + 1.0 μ M NAA

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DEDICATION

This dissertation is dedicated to the late Norman E. White, Jr. (1935-1987). He was a consummate educator who made a lasting impression on the people who were fortunate enough to have known him. He was also a devout friend and is missed dearly.

ABSTRACT

Red pine seedlings alternate between root and shoot production in a periodic fashion. This habit is referred to as episodic growth and is assumed to be controlled by endogenous plant growth regulators. This project was concerned with the role played by cytokinins in the control of episodic growth.

After confirming the existence of episodic growth in red pine seedlings, a carrier solution was optimized and a series of exogenous application trials was conducted. Only those solutions containing a surfactant and N⁶-benzylaminopurine (BAP) produced an observable response. Foliar applications of BAP induced the formation of an apical cluster of buds. As the BAP concentration was increased from 2.5 to 200 mg/l, there was a corresponding increase in the number of buds formed. Hypocotyl length and the percentage of the induced buds that elongated were inversely proportional to the BAP concentration used. The response to BAP, as affected by seedling age and application number, was also determined. The younger the seedlings were when they received their first BAP treatment and the more applications they received, the greater the reduction in hypocotyl growth. As the number of BAP applications increased, the number of buds formed was proportionately greater. As the age at which seedlings received their first treatment of BAP increased, the percentage of buds that elongated decreased. When the same carrier solution, containing 200 mg/l BAP, was applied to the potting mix in which the seedlings were grown, axillary bud development along the hypocotyl was promoted. Germinating seeds were also treated with the same solution. Longer exposures to BAP resulted in progressively shorter hypocotyls and needles and in lower survival.

The origin of the buds induced by BAP was characterized histologically. The BAP treatments were found to induce precocious axillary bud development and

adventitious bud formation. The suitability of this bud cluster for micropropagation of red pine was also explored.

Because the CKs produced by red pine have never been identified, they were qualitatively characterized. The cytokinins produced by roots and shoots were identified based on the retention times of biological activity on Sephadex LH-20 and Spherisorb ODS-2. Zeatin and zeatin riboside (ZR) were both found in the basic fraction of root and shoot extracts. The presence of ZR in both tissues was confirmed by GC-MS analysis. Evidence for the presence of dihydrozeatin O-glucoside in the shoot basic fraction and for zeatin O-glucoside in the roots is also presented. Zeatin riboside monophosphate, isopentenyladenosine monophosphate (iPMP) and glucosyl phosphate derivatives were found in both root and shoot acidic fractions. The presence of iPMP in both tissues implies that cytokinin biosynthesis is occurring throughout the plant. Evidence suggesting the presence of a phosphorylated derivative of zeatin O-glucoside in the roots is presented.

GENERAL INTRODUCTION

Seedling Growth and Establishment

Red pine (*Pinus resinosa* Ait.) is a commercially important species found throughout the north central and northeastern United States. Unfortunately, attempts at reestablishing this species are frequently met with failure. Red pine growers agree that larger seedlings are needed to improve outplanting survival (Eggleston and Sharp, 1986). At present, underdeveloped red pine seedlings must be kept in the greenhouse or nursery bed longer than related species in order to achieve greater height growth. If smaller stock is outplanted, there is a greater need to control competing vegetation through the use of herbicides and to protect seedlings from herbivory. These mitigating measures are expensive and their costs justify searching for treatments that will enhance height growth.

Red pine is a fixed grower, which means that all stem units that elongate in the current year are laid down in the terminal bud during the previous growing season (Doak, 1935; Duff and Nolan, 1958; Sucoff, 1971). Although height growth is influenced by the extent to which the preformed stem units elongate, and, therefore, by the environmental conditions during the current year, differences in annual shoot increment are primarily a function of the number of stem units formed in the terminal bud during the previous growing season (Lanner, 1976).

Stem unit number can be altered by increasing the rate of stem unit initiation (i.e., by shortening the plastochron, the time interval between initiation of consecutive primordia) or by increasing the length of the initiation period. Sucoff (1971) estimates that the red pine plastochron varies from greater than one day for sterile scale initiation in the early spring to about three hours during mid-

summer. No attempts have been made to alter the plastochron experimentally so nothing can be said about the possibility of shortening it. However, it may be possible to increase height growth by lengthening the period during which stem units are initiated.

It is known that red pine needle length is positively correlated with moisture supply (Strothmann, 1967; Glerum and Pierpoint, 1968; Clements, 1970). Sucoff (1971) has also demonstrated a positive relationship between rates of needle elongation and stem unit formation. Paterson (1987) went on to explain that because the rate of needle elongation reaches a maximum when buds are formed and because both processes are sensitive to moisture stress, the former can be used to predict the level of bud development. Therefore, needle length can be used to judge the potential of various treatments to produce greater height increments.

Because the extent of root development controls the rate of water absorption and needle elongation is sensitive to moisture supply, needle length is also indicative of root development. Paterson and Fayle (1984) demonstrated that the proportion of needles flushed and their length at the end of the growing season were highly correlated with root activity. Considering that a seedling's capacity to produce new roots after outplanting is related to its early survival and growth (Stone, 1955; Burdett, 1979; van den Driessche, 1983), Paterson and Fayle used the proportion of needles flushed and their length at the end of the growing season as a means of predicting the degree of seedling establishment.

Episodic Growth

Based on the foregoing evidence and red pine's growth pattern, it would appear that root activity influences shoot growth by doing more than just improving

the seedling's water and nutrient absorption capacity. It is thought that roots produce chemical signals that are involved in the control of shoot development. Drew (1982) provided indirect evidence for this claim when he demonstrated that red pine seedlings alternate between root and shoot production throughout the growing season (Drew, 1982). This behavior, he contends, allows the seedling to maintain a functional equilibrium between its roots and shoots. This same growth pattern has been observed in other species of pine (Ledig et al., 1976; Drew and Ledig, 1980) and is referred to as episodic growth. Even though surges in root and shoot growth have never been correlated with changes in endogenous levels of any of the plant growth regulators (PGRs), they have been implicated in the control of episodic growth, through circumstantial evidence (Drew, 1982).

Objectives of the Study

Cultural treatments are used by growers to alter seedling development in an attempt to produce seedlings better adapted for survival on a given site (Duryea, 1984; Iverson, 1984). Although there may be a physiological basis for these treatments, they are often developed through a process of trial and error. If the internal mechanism by which seedlings control their own growth and development was better understood, it might be possible to devise more effective strategies for manipulating seedling development in order to enhance seedling establishment. With this in mind, the overall goal of the project begun by the research described in this dissertation is to understand the role PGRs play in the control of red pine seedling development. More specifically, this research is concerned with the role cytokinins (CKs) play in the control of episodic growth.

Explanation of the Alternate Dissertation Format

Before characterizing the endogenous CKs of red pine, it was first necessary to confirm the existence of episodic growth (Section I). Once this was accomplished, evidence was needed for the involvement of CKs in the control of shoot development. For this purpose, a carrier solution was optimized and a series of exogenous application trials was undertaken (Section II). Cytokinin treatment did influence shoot development, but not in the expected manner. Instead of promoting shoot growth, exogenously applied CKs induced the formation of a terminal cluster of adventitious buds and provoked a short-lived outgrowth of existing primordia. The origin of these buds was characterized histologically (Appendix A), and their suitability for the clonal propagation of red pine was explored (see Appendix B).

Once it was shown that CK treatment altered shoot development, the next step was to search for a correlation between endogenous CK levels and surges in shoot growth. However, because the CKs produced by red pine, or any other pine, have never been identified unambiguously, qualitative characterization had to precede quantitative determinations. Section III reports on the CKs identified in the shoots of red pine and Section IV reports on the CKs identified in the roots of red pine.

Apart from the gas chromatography-mass spectrometry work, which was conducted by Mr. J. K. Heald (Technician, Department of Biological Sciences, University College of Wales, Aberystwyth, Dyfed, Wales, U. K.), all other work described in this dissertation was performed by the first author (the Ph.D. candidate) of all four publications.

LITERATURE REVIEW

Early work in the area of plant tissue culture has provided convincing evidence for the involvement of CKs and auxin in the control of plant growth and morphogenesis. Although direct evidence is still lacking, the examination of two other experimental systems has led to complementary results. Using the moss *Physcomitrella patens* and the organism responsible for crown gall disease, *Agrobacterium tumefaciens*, workers have been able to demonstrate a correlation between plant morphogenesis and endogenous levels of CK and auxin. The major findings in these three areas are summarized in the remainder of this section.

Exogenously-applied PGRs Control Plant Development

In what is now considered a classic experiment, Skoog and Miller (1957) demonstrated that quantitative interactions between CK and auxin provide a mechanism for the regulation of plant organogenesis. When undifferentiated tobacco pith callus was cultured on an agar-based medium containing 2 mg/l indole acetic acid (IAA, an auxin) and 0.02 mg/l kinetin (a CK), root primordia were initiated in the callus. As CK levels were increased, while holding the auxin level constant, the callus went through a phase of rapid, undifferentiated growth. Eventually, with further increases in CK (to between 0.5 and 1.0 mg/l), shoot primordia were initiated in the callus. This landmark discovery has become the basis for virtually all plant tissue culture being done today. However, tissue culture studies constitute a form of exogenous application and do not provide conclusive evidence for the developmental roles of PGRs.

Criticism of Exogenous Application Experiments

The simplest way to determine if a substance is involved in the control of a developmental event is to provide an exogenous supply and observe the plant's response, such as was done by Skoog and Miller (1957). However, the results of exogenous application studies using PGRs are difficult to interpret because so little is known about mechanisms of uptake, transport, inter- and intracellular localization, the responsiveness of cells and tissues to PGRs, and metabolic pathways leading to activation and catabolism (Medford et al., 1989). Due to the wide range of possible fates, physiologically unrealistic concentrations of an exogenously-applied PGR are needed to elicit the response to which it is attributed.

This observation has led to a controversy within the field of PGR research. On one side there are those who believe that because dose-response curves for exogenously-applied growth substances can span four to six orders of magnitude, whereas changes in the corresponding endogenous compounds are rarely outside the two to 10-fold range, the morphogenetic response seen is most likely due to a change in the plant's sensitivity to the substance rather than a change in its concentration (Trewavas, 1983). In an opposing argument, Horgan (1987) wrote: "Given the multitude of biochemical events that may attenuate the concentration of an externally-applied hormone before it reaches its site of action, it would seem naive to believe that the dose-response relationships for endogenous compounds will have the same logarithmic forms as those for the externally-applied compounds."

One way to resolve this hotly contested issue is to induce physiologically realistic increases in the level of a given PGR *in vivo* and observe its developmental effect. Prior to the recent molecular biological revolution, this approach was not technically possible in higher plants. In the interim, classical genetic studies

using bryophyte mutants have provided some insight into the roles played by CK and auxin in the control plant development.

The Use of Bryophytes as a Model System

Plant growth regulators such as cytokinin and auxin are known to be synthesized by microorganisms as products of secondary metabolism (Sembdner and Gross, 1986). However, there is no evidence that these chemicals play any regulatory role. It is generally believed that in contrast to animal systems, where the steroid and peptide hormones co-evolved with their receptors, PGRs seem to have become incorporated into the plant's regulatory system following receptor evolution. The bryophytes appear to be the simplest plant in which such a regulatory system evolved. Because of their relative simplicity, it is easier to study cytokinin and auxin mode of action in mosses first and then apply this knowledge to higher plants (Johri, 1988).

The moss *Physcomitrella patens* is frequently used to investigate growth regulation and development because it is technically convenient for doing physiological and classical genetics studies. The normal gametophytic development of *P. patens* initially involves the growth of branched filaments called chloronemata. This stage is followed by the production of a second type of tissue, caulonemata, that consists of long, radial, peripheral filaments of tubular cells which are longer and thicker than the chloronemal cells. Together the caulonemata and chloronemata constitute the protonema. Eventually, caulonemal side branches give rise to gametophores, consisting of a stem and leaves. When wild-type (W-T) strains of *P. patens* were treated with naphthalene acetic acid (NAA, an auxin), gametophore production was decreased, some remained in the bud stage, and many short, thick rhizoids arose from their surfaces. In contrast, treatment of

the W-T strain with 6-benzylaminopurine (BAP) induced a massive increase in gametophore production. The same BAP concentration that induced this response also inhibited rhizoid production (Ashton et al., 1979a).

A series of *P. patens* mutants altered in their response to CKs and auxin have been isolated by Ashton et al. (1979a). Clones derived from chemically mutagenized spores, and screened for growth on high concentrations of auxin and/or CK, fell into three distinct categories, those resistant to: 1) both BAP and NAA, 2) only NAA and 3) only BAP. Those mutants that were resistant to exogenously-supplied BAP exhibited a normal phenotype only when pre-treated with auxin. Similarly, those mutants that were resistant to auxin were repaired by an exogenous supply of BAP. The authors reasoned that CK-resistant mutants were deficient in their auxin biosynthetic pathway and that the auxin-resistant mutants were incapable of CK biosynthesis. Based on their findings, the authors proposed a model in which cytokinin sensitivity was auxin-dependent and auxin sensitivity was CK-dependent. The authors concluded that both CKs and auxin were necessary for normal gametophytic development.

In another report, Ashton et al. (1979b) described the isolation of several distinct classes of *P. patens* mutants (OVE) which overproduce gametophores. When cultured on minimal media, these OVE mutants resembled the W-T strain grown in the presence of BAP (i.e., overproduction of gametophores). The authors suggest that the OVE mutants overproduce endogenous CK(s) as a result of either an increase in the rate of biosynthesis or a decrease in the rate of degradation. Comparative analysis revealed 25-100 times more isopentenyl adenine (iP) in the culture medium of OVE than was present in the medium on which the W-T strain was grown (Wang et al., 1981a). When the OVE mutants were fed [8-¹⁴C]adenine, radioactively-labelled iPA was detected in the culture medium and

labelled isopentenyl adenosine (iPA) and its nucleotide were detected in the gametophytic tissue (Wang et al., 1981b). Unpublished work on CK catabolism detected no difference between W-T and OVE strains (Cove and Ashton, 1984). Thus it appears that gametophore overproduction is in fact due to CK overproduction.

To date, no auxin-overproducing *P. patens* mutants have been isolated. However, using a gas chromatography-mass spectrometry (GC-MS) technique developed by Magnus et al. (1980), which includes 4,5,6,7-tetradeutero-indole-3-acetic acid as an internal standard, Ashton et al. (1985) have detected indole-3-acetic acid (IAA, an auxin) in *P. patens* protonemal tissue. In another moss, *Funaria hygrometrica*, an inverse relationship was detected between caulonemata production and IAA oxidase activity (Johri, 1974, 1975; Johri and Desai, 1973). Because IAA stimulates caulonemata production, it seems likely that moss morphogenesis is controlled, in part, by modulating rates of hormone degradation through the activity of catabolic enzymes (Cove and Ashton, 1984).

The Use of Crown Gall for Studying Plant Morphogenesis

Recent advances in plant molecular biology have furnished techniques for studying morphogenesis in higher plants. Crown gall (a disease caused by *Agrobacterium tumefaciens*) provides a convenient means of directing endogenous PGR biosynthesis. By generating a series of mutant strains through transposon insertion within the transfer deoxyribonucleic acid (T-DNA) region of the tumor-inducing (Ti) plasmid, it was possible to induce the formation of abnormal crown gall tumors on infected plants. When an insertion occurred within what is now referred to as the *tmr* region, the resulting mutant strain incited tumors with roots proliferating from the tumor callus. A mutant strain resulting from an

insertion within what is now known as the *tms* region of the T-DNA incited tumors with shoots growing from the tumor callus (Garfinkel et al., 1981; Ooms et al., 1981).

Using radioimmunoassay, Akiyoshi et al. (1983) measured IAA and total zeatin riboside-like CK levels in tumors incited on tobacco stems by W-T, *tms* mutant and *tmr* mutant strains of *A. tumefaciens*. Tumors incited by the *tmr* mutant contained about 129 picomoles IAA /gram fresh weight callus (pmol IAA/g FW) and 0.54 pmol zeatin riboside (ZR)/g FW, leading to a ZR:IAA ratio of 0.004. Tumors incited by the *tms* mutant strain contained about 1,400 pmol ZR/g FW and 70 pmol IAA/g FW, yielding a ZR:IAA ratio of 20. Tumors induced by W-T strains contained about 48 pmol ZR/g FW and 295 pmol IAA/g FW (ZR:IAA=0.16), whereas uninfected plants contained about 0.97 pmol ZR/g FW and 128 pmol IAA/g FW (ZR:IAA=0.001).

Barry et al. (1984) were the first to show that the *tmr* locus encodes the genetic information for isopentenyl transferase (ipt), the enzyme responsible for catalyzing the first step in CK biosynthesis (Chen, 1982). This work was independently confirmed by Akiyoshi et al. (1984) and Buchmann et al. (1985). Subsequently it was shown that two other genes within the T-DNA region encode enzymes catalyzing the biosynthesis of auxin (Schroder et al. 1984; Van Onckelen et al., 1985; and Van Onckelen et al., 1986).

As anticipated, interrupting the gene which encodes ipt (i.e., the *tmr* mutation) led to tumors with lower levels of ZR and normal levels of IAA. The reduced levels of IAA in tumors incited by *tms* mutants were also predictable. However, the tremendous increase in ZR levels in the same tumors was completely unexpected. This suggests that the change in tumor morphology may be more a function of CK concentration than IAA concentration.

Recently, by means of GC-selected ion monitoring-MS using ^{15}N - and ^3H -labelled internal standards, McGaw et al. (1988) quantified all of the major CKs produced in tobacco crown gall tumors incited by W-T, *tms* mutant and *tmr* mutant strains of *A. tumefaciens*. From their detailed analysis of the major CK metabolites, it was concluded that the increased CK levels seen by Akiyoshi et al. (1983) were a result of a reduction in CK side-chain cleavage. This conclusion was confirmed by Palni et al. (1988), who, using partially purified enzyme extracts, demonstrated that cytokinin oxidase activity is inhibited by auxin.

Transforming Higher Plants with PGR Biosynthetic Genes

The cytokinin biosynthetic gene

During crown gall infection, any DNA present between the direct repeat border sequences of the T-DNA region on the Ti plasmid is randomly inserted into the plant genome (Holsters et al., 1983; Wang et al., 1984). For example, by removing all DNA but those sequences which encode *ipt*, this gene alone will be inserted into the plant. Thus, in addition to providing a means of directing synthesis of CK and auxin, *A. tumefaciens* can be used as a vehicle for introducing foreign genetic material into plants (Klee et al., 1985). However, the inserted gene would be expressed constitutively by *Agrobacterium*'s weak promoter.

The *Agrobacterium* genes encoding the enzymes responsible for CK and auxin biosynthesis have been placed under the control of a strong, constitutively-expressed plant promoter in petunia (*Petunia hybrida*). The effects of CK and auxin overproduction were dramatic. Transgenic CK-overproducing petunias were difficult to regenerate and were incapable of rooting. The plants that did survive showed a lack of apical dominance and were very green. These effects

were consistent with roles assumed to be played by CKs in the control of plant development. The responses of transgenic auxin-overproducing petunia plants were also consistent with its assumed roles: complete apical dominance, greater internode length, leaf curling, a doubling of xylem and phloem production, and parthenocarpic fruit production (Klee et al., 1987a). These results provided additional supportive evidence for the roles played by CK and auxin but a regulated system of synthesis was needed to provide definitive proof of the roles played by CK and auxin.

The heat shock genes provide such a system. Under conditions of elevated temperatures (usually 10-40°C above normal) plants rapidly switch from transcription and translation of normal messenger ribonucleic acid (mRNA) to heat shock mRNAs. The resulting proteins impart thermotolerance of what would otherwise be lethal temperatures (Key et al., 1987). The promoter providing for thermal induction in *Zea mays* (hsp 70) has been isolated by Rochester (1986). This promoter has recently been fused to the auxin and CK biosynthetic genes of *Agrobacterium* and used to transform tobacco (*Nicotiana plumbaginifolia*) and *Arabidopsis thaliana* (Medford et al., 1989).

Heat induction of the transgenic tobacco plants caused *ipt* mRNA to accumulate and led to the following increases in endogenous CKs: zeatin (Z), 52-fold; ZR, 23-fold; and zeatin riboside 5'-monophosphate (ZMP), two-fold. At control temperatures, ZR and ZMP increased to levels three and seven times W-T levels, respectively, indicating that the chimeric gene is expressed to some extent even in the uninduced transgenic plants. Despite the differences between the induced and uninduced transgenic plants, increased CK levels in both produced the same effects, namely, release of axillary buds, reduced stem growth and leaf area, and an underdeveloped root system. These results suggest a threshold above which

additional CK has no further effect on development.

The auxin biosynthetic genes

Unlike CK biosynthesis, the pathway employed by *Agrobacterium* to synthesize auxin is different from that used by plants. In *Agrobacterium*, there are two gene products involved in auxin biosynthesis. The product of the first gene, *iaaM*, converts tryptophan to indoleacetamide (IAM); the second gene (*iaaH*) product converts IAM to IAA. Klee et al. (1987b) fused the first gene to the cauliflower mosaic virus (CaMV) 19S promoter and used this construct to transform petunia. Although the CaMV promoter is constitutively expressed in petunia, plants normally do not contain IAM; therefore, an auxin-induced response was not expected. However the 19S/*iaaM* transformants exhibited characteristics normally attributed to a change in the concentration of endogenous auxin. When IAA levels were measured in these transformants, they were found to be 10 times those found in the control tissue. Although the mechanism by which these plants converted IAM to IAA is still unknown, the authors maintain that their data support the notion that absolute amounts of endogenous auxin are responsible for auxin's control over development.

Thus, regulation of CK and auxin biosynthesis at the genetic level confirms earlier evidence for the role CKs and auxin play in the control of plant development.

The Decision to Analyze for Cytokinins

Obviously, identifying endogenous PGRs in higher plants, including trees, is a crucial step in studying their developmental roles. Because PGRs occur in such minute quantities and are often difficult to separate from other components present in plant extracts, specialized physicochemical techniques have been devel-

oped for isolating, identifying and quantifying PGRs. The time and effort required to conduct these analyses has, in many cases, forced researchers to concentrate on a single family of PGR when assessing the developmental roles played by PGRs.

It would appear that, at least in red pine, shoot and root development are inextricably linked. Cytokinins, which are assumed to be synthesized in root tips (Radin and Loomis, 1971; Engelbrecht, 1972; Short and Torrey, 1972; Feldman, 1975; Skene, 1975; Van Staden and Davey, 1979) and exported to the shoot in the xylem, are thought to be involved in the regulation of shoot development (Burrows and Carr, 1969; Alvim et al., 1976; Purse et al., 1976; Mozes and Altman, 1977; Van Staden and Davey, 1979). Ironically, CKs are also potent growth inhibitors of the tissue within which they are produced, the roots (Darimont et al., 1971; Scott, 1972; Svensson, 1972; Goodwin, 1978). Because of our interest in root development and because the effect of CKs on roots and shoots is interrelated, it was decided to assess the role CKs play in the control of episodic growth in red pine seedlings.

Cytokinin Diversity

Cytokinins, unlike most other classes of PGRs, are defined physiologically rather than structurally. It is universally agreed that "a cytokinin is best defined as a compound which, in the presence of optimal auxin, induces cell division in tobacco pith or similar tissue cultures (e.g., soybean callus, carrot secondary phloem); in its other activities a cytokinin also resembles kinetin, the first known cytokinin" (Letham, 1978). With few exceptions most naturally-occurring cytokinins are N⁶-substituted adenine derivatives. The two major exceptions are dihydroconiferyl alcohol and N,N'-diphenylurea. The former, originally found in

maple (Granath and Schuerch, 1953), is not active in all cytokinin bioassays (Lee et al., 1981). Hence, it is not considered universally to be a CK. Diphenylurea, on the other hand, is known to suppress the activity of CK oxidase, the primary catabolic enzyme of CKs. Therefore, it may mimic CK activity by reducing CK catabolism.

It is generally agreed that the primary pathway for CK biosynthesis in plants is through the condensation of isopentenyl pyrophosphate and adenosine 5'-monophosphate (AMP), to form isopentenyl adenosine 5'-monophosphate (iPMP) and pyrophosphate (McGaw, 1987). The 5' phosphate and the ribosyl moieties are subsequently cleaved, enzymatically, to form isopentenyl adenosine (iPA) and iP, respectively. The latter three compounds (iPMP, iPA and iP) constitute the first major class of CKs found in plants, the iP derivatives. Both iP and iPA can be stereospecifically hydroxylated at the terminal methyl group in the *trans* position of the side chain (Miura and Hall, 1973; Einset and Skoog, 1973; Laloue et al., 1977), giving rise to the second major class of CKs, zeatin and its derivatives (e.g., Z, ZR, etc.). Side-chain saturation of Z-type CKs results in the corresponding dihydrozeatin derivatives (Sondheimer and Tzou, 1971; Palmer et al., 1981), the third major class of CKs.

In addition to CK-ribosides, glucosides and amino acid conjugates have been reported. A glucosyl moiety can be conjugated, via a β -D linkage, to the hydroxyl group on the side chain (Horgan, 1975; Parker et al., 1978), or to the 3, 7 or 9 nitrogen atoms of the purine ring (Parker and Letham, 1974; Parker et al., 1973; Letham et al., 1975; Entsch et al., 1979). There is even evidence for an unusual CK metabolite in which a glucosyl moiety is attached to a ribosyl moiety which is, in turn, attached to N⁹ of the purine ring (Taylor et al., 1984). Finally, an N⁹ alanyl derivative of zeatin has been isolated from lupine (Parker et al., 1978).

The Necessity of Qualitative Characterization

There is now evidence for 38 naturally-occurring CKs (Qamaruddin, 1989a). No one plant is known to be capable of synthesizing all 38 but each one produced is thought to serve a specific function. For example, the CK-glucosides are considered to be storage forms, the CK-ribosides are considered to be transport forms and the free bases are considered to be active forms of CK (Van Staden and Davey, 1979; Laloue and Pethe, 1982). In *Zea mays*, Z, ZR, zeatin O-glucoside, zeatin riboside O-glucoside, dihydrozeatin O-glucoside, and dihydrozeatin riboside O-glucoside have been identified using physicochemical techniques. Therefore, in order to obtain meaningful results, the endogenous CKs must be qualitatively characterized before attempting quantitative determinations.

Evidence for CKs Controlling Pine Morphogenesis

Although all plants are assumed to contain CKs, the number of CKs unambiguously identified in higher plants is relatively few (see Horgan, 1984 for a listing). Most of the reports describing the existence of CKs in pine extracts are based on the presence of biological activity in an extract or the detection of biological activity with the same chromatographic properties as known CKs. For example, two early reports described how crude extracts from the seed of *Pinus banksiana* stimulated the growth of carrot tissue (Mauney et al., 1952) and pine tissue (Loewenberg and Skoog, 1952). Later, using a tobacco bioassay described by Rogozinska et al. (1965), CK-like activity was detected in strips of chromatographic paper used to separate the components of crude extracts derived from several tissues of Scots pine (*Pinus sylvestris*; Rogozinska, 1967).

Pine extracts contain a variety of unknown compounds that are difficult to separate from CKs and which interfere with their characterization (see Discus-

sion, Section III of this dissertation). Moreover, the equipment required for unequivocal indentifications (i.e., a mass spectrometer) is inaccessible to many of the researchers engaged in this work. Therefore, very little progress has been made in identifying and measuring CKs in pines.

Recently, immunological assay procedures have been used to substitute for more rigorous identification and quantification techniques (Weiler, 1980; Weiler and Spanier, 1981). This approach has the advantages of being rapid and relatively inexpensive and has the potential to be both accurate and precise. However, immunoassays are of limited value unless they have been substantiated using more stringent methodologies. Furthermore, because of the possibility of interference by unknown compounds in crude extracts and because antibodies raised against a given CK often have a high degree of cross-reactivity with a range of endogenous CKs, immunoassays should be performed following chromatographic purification. Both high-performance liquid chromatography (Badenoch-Jones et al., 1984; MacDonald and Morris, 1985; Von Schwartzberg et al., 1988) and immunoaffinity chromatography (MacDonald et al., 1981; MacDonald and Morris, 1985; Hansen et al., 1989) have been used for preliminary purification of the extracts.

With the introduction of immunoassays, there has been renewed interest in the effect of CKs on pine seedling development. Haggman (1989) analyzed the total Z-type CKs in partially purified extracts from Scots pine buds during springtime, using an enzyme-linked immunosorbent assay (ELISA). He found that renewed bud growth was positively correlated with Z-type CK concentration. In an unrelated study, but using a similar approach, Qamaruddin et al. (1989b) detected an increase in both iPA- and ZR-type CKs just prior to bud burst in Scots pine. In the same study, an exogenous supply of CK stimulated premature

bud break. The latter group also showed a transient increase in both types of CK in needles prior to the increase in bud CKs. They suggested that the needles may be supplying CK to the buds.

Finally, in a separate report, Qamaruddin (1989a) used an ELISA system to measure iPA- and ZR-type CKs in the roots and shoots of Scots pine seedlings during the induction of and recovery from water stress. His results revealed reductions in both CK types, in both tissues, that were proportional to the severity and duration of the imposed stress. In contrast, only ZR-type CK levels increased in the needles during the stress-recovery period. As part of the same study, exogenous applications of CK increased transpiration rates in stressed seedlings. The author maintains that, taken together, these findings imply that CKs could be part of the mechanism responsible for the control of transpiration. More generally, he hypothesizes that CKs are involved in the transition from growth arrest to growth activity.

**SECTION I. CONFIRMATION OF EPISODIC GROWTH IN RED
PINE SEEDLINGS**

INTRODUCTION

Episodic growth is a phrase used to describe the way in which seedlings alternate between root and shoot production throughout the growing season. This was first reported in the genus *Pinus* by Drew and Ledig (1980) using loblolly pine (*Pinus taeda* L.). Subsequently, Drew (1982) demonstrated the existence of episodic growth in red pine (*Pinus resinosa* Ait.). Before devoting the effort required to completely characterize the cytokinins (CKs) in red pine, it was first necessary to verify that red pine seedlings grow episodically. This section reports on a growth study which was conducted to confirm the results of Drew (1982).

MATERIALS AND METHODS

Seedling Culture

Red pine seeds were surface-sterilized by stirring them in a 10% aqueous solution of commercial bleach (5.25% sodium hypochlorite before dilution) for 10 minutes, followed by one rinse with 0.01 N HCl and three rinses with sterile, deionized distilled water. The surface-sterilized seeds were rinsed overnight with tap water in a beaker covered with cheesecloth and then germinated on sterilized blotter paper saturated with autoclaved deionized, distilled water. After the appearance of a radicle hook, the seeds were individually planted in four-cubic-inch (65.5 cm³) Leach Pine Cells (Ray Leach Conetainer Co., Canby, OR, USA, 97013) filled with Terra-lite Forestry Mix (a 1:1 commercial potting mixture of peat and vermiculite pH-adjusted for conifer growth, W. R. Grace Co., Cambridge, MA, USA, 02140) at field capacity. Three weeks after sowing, fertilization was begun. Seedlings were treated weekly, for four weeks, using Peters "Conifer Starter" (7-40-17, W.R. Grace Horticultural Products, P.O. Box 238, Fogelsville, PA, USA, 18051) diluted to 66, 165 and 132 mg/l N, P and K, respectively. Thereafter, Peters "Conifer Grower" (20-7-19), diluted to 187, 29 and 140 mg/l N, P and K, respectively, was used for the weekly fertilization. All seedlings were grown in a greenhouse under 18 hour photoperiods, with incandescent (200 watt Sylvania Excel-line) and fluorescent (40 watt Philips-Westinghouse cool white) lamps used to extend daylengths (average photon flux density of 8.6 $\mu\text{mole/m}^2/\text{s}$). Monthly average daytime temperatures ranged between 25 and 30°C, nighttime temperatures ranged between 15 and 20°C. Seedlings were watered daily with deionized, distilled water.

Experimental Protocol

One month after planting, 20 seedlings were harvested. The potting mix was carefully washed away from the root system of each seedling and the roots were separated from the shoots by severing the stem one cm above the first lateral root. The separated plant parts were oven-dried for 24 hr. at 70°C. After removal from the oven, the seedling parts were allowed to cool *in vacuo* over Drierite for 24 hr. before weighing. As the seedlings got larger, progressively longer drying times were used. This process was repeated at weekly intervals for a total of 26 weeks. After the fifteenth harvest, it was decided to remove the leaves from the stems before drying, so oven-dry weights (DWs) could be obtained for leaves, stems and roots separately. However, the sample size was the same ($n=20$) for the remainder of the study.

RESULTS

The increase in average oven-dry weight of each plant part over the average dry weight of the corresponding part from the previous week was expressed as a percent of the increment in total seedling DW during the same interval. A graph showing the allocation of DW increment to the roots and shoots for the first 16 weeks of the study is shown in Figure 1; the distribution of DW increment to the leaves, stems and roots over the final 10 weeks of the study is plotted in Figure 2.

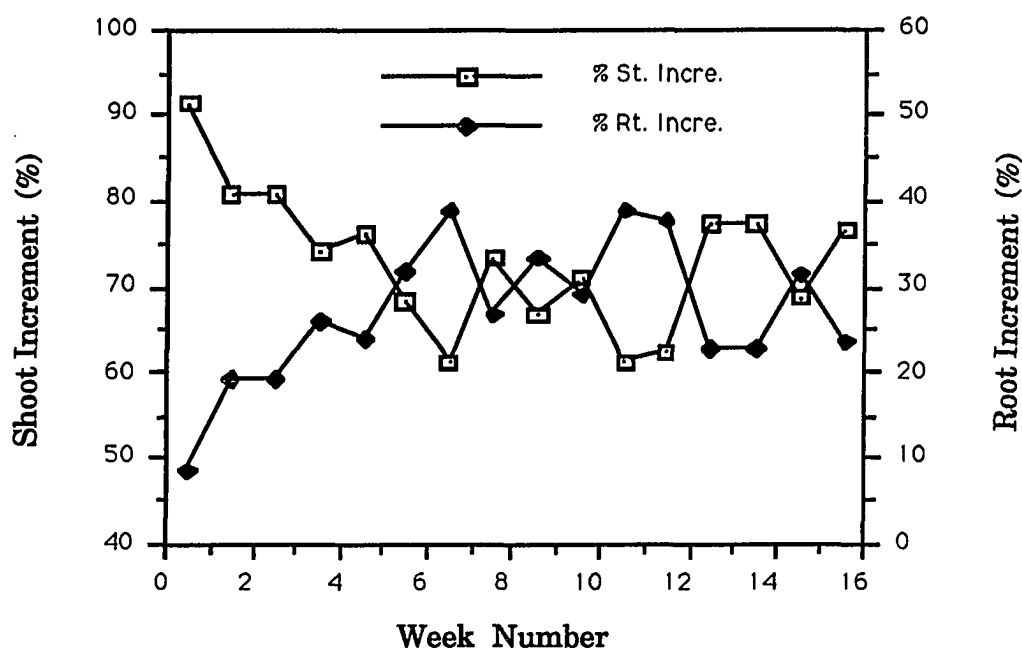


Figure 1. Allocation of weekly DW increment to the shoots and roots of red pine seedlings. Shoot and root dry weights were taken at one-week intervals for each of 20 seedlings. Individual points were obtained by subtracting the previous week's average shoot and root dry weights from their corresponding values in the current week and dividing by the total (shoot + root) dry weight increment for the same period

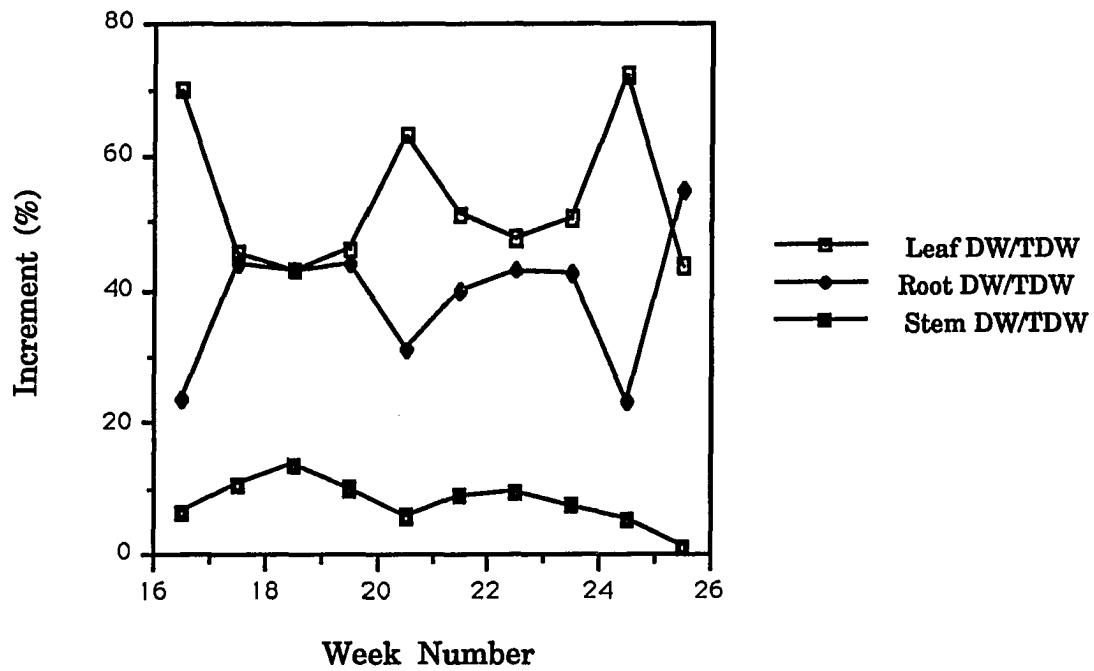


Figure 2. Distribution of weekly DW increment to the leaves, stems and roots of red pine seedlings ($n=20$). Calculations were performed in an analogous manner to those described in Figure 1

DISCUSSION

Whether looking at only roots and shoots (Figure 1) or leaves, stems and roots (Figure 2), it is obvious that seedlings do not allocate a fixed percentage of their weekly biomass increment to the various plant parts. Instead, as shown by Drew (1982), the seedlings alternate between favoring shoots (leaves) or allocating roughly equivalent amounts of carbohydrate to both roots and shoots in a very cyclic manner. These results confirm the existence of episodic growth in red pine seedlings.

LITERATURE CITED

- Drew, A. P. 1982. Shoot-root plasticity and episodic growth in red pine seedlings. *Annals of Botany* 49: 347-357.

**SECTION II. MANIPULATING RED PINE SEEDLING DEVELOPMENT
THROUGH EXOGENOUS APPLICATIONS OF BENZYL-
AMINOPURINE**

ABSTRACT

Red pine (*Pinus resinosa* Ait.) seedlings received exogenous applications of 6-benzylaminopurine (BAP) in an attempt to modify shoot development. A range of carrier solutions was tested; only those carriers containing a surfactant (Tween 20) and BAP produced observable responses. Foliar applications of BAP induced the formation of an apical cluster of adventitious buds. As the BAP concentration was increased from 2.5 to 200 mg/l, there was a corresponding increase in the number of buds formed. Hypocotyl length and the percentage of the induced buds that elongated were inversely proportional to the BAP concentration used. The response to BAP, as affected by seedling age and application number, was also determined. The younger the seedlings were when they received their first BAP treatment and the more applications they received, the greater the reduction in hypocotyl growth. A greater number of BAP treatments also induced the formation of proportionately more adventitious buds. As the age at which seedlings received their first treatment of BAP increased, the percentage of buds that elongated decreased. When the same carrier (an aqueous solution of Tween 20), containing 200 mg/l BAP, was applied to the potting mix, instead of directly on the seedlings, axillary bud development was promoted along the length of the hypocotyl. Germinating seeds were also treated with the same solution. Longer exposures to BAP resulted in progressively shorter hypocotyls and needles and lower survival.

INTRODUCTION

Red pine (*Pinus resinosa* Ait.) seedling growers agree that a means of producing larger container stock is needed to improve field survival (Eggleston and Sharp, 1986). At present, underdeveloped container seedlings must be kept in the greenhouse or a nursery bed longer to achieve greater height growth. If the smaller stock is outplanted, there is a greater need to control competing vegetation through the use of herbicides and to protect the seedlings from herbivory. These mitigating measures are expensive and their costs justify the effort expended in search of treatments that will enhance height growth.

Red pine is a fixed grower, which means that all stem units that elongate in the current year are laid down in the terminal bud during the previous growing season (Doak, 1935; Duff and Nolan, 1958; and Sucoff, 1971). Differences in annual shoot increment are primarily a function of the number of stem units in the terminal bud. Although no attempts have been made at altering the rate of stem unit initiation, it may be possible to increase height growth by lengthening the period during which stem units are initiated.

It is known that red pine needle length is positively correlated with moisture supply (Strothmann, 1967; Glerum and Pierpoint, 1968; Clements, 1970). Sucoff (1971) has also demonstrated a positive relationship between rates of needle elongation and stem unit formation. Paterson (1987) went on to explain that because the rate of needle elongation reaches a maximum at the time during which buds are formed and because both processes are sensitive to moisture stress, the former can be used to predict the level of bud development. Therefore, needle length can be used to judge the potential of various treatments to produce greater height increments.

Because the extent of root development controls the rate of water absorption and needle elongation is sensitive to moisture supply, needle length is also indicative of root development. Paterson and Fayle (1984) demonstrated that the proportion of needles flushed and their length at the end of the growing season were highly correlated with root activity. Considering that a seedling's capacity to produce new roots after outplanting is related to its early survival and growth (Stone, 1955; Burdett, 1979; van den Driessche, 1983), Paterson and Fayle (1984) used the proportion of needles flushed and their length at the end of the growing season as a means of predicting the degree of seedling establishment.

Based on the foregoing evidence and red pine's growth pattern, it would appear that root activity influences shoot growth by doing more than just improving the seedling's water and nutrient absorption capacity. It is thought that roots produce chemical signals that are involved in the control of shoot development. Drew (1982) provided indirect evidence for this claim when he demonstrated that red pine seedlings alternate between root and shoot production throughout the growing season (Drew, 1982). This behavior, he contends, allows the seedling to maintain a functional equilibrium between its roots and shoots. This same growth pattern has been observed in other species of pine (Ledig et al., 1976; Drew and Ledig, 1980) and is referred to as episodic growth. Even though surges in root and shoot growth have never been correlated with changes in endogenous levels of any of the plant growth regulators (PGRs), they have been implicated in the control of episodic growth, through circumstantial evidence (Drew, 1982).

Because PGRs are active in such minute quantities, extraction, purification and quantification procedures are difficult to perform. As a result, researchers working in this field generally confine their efforts to a single group of PGR. We have chosen to work with cytokinins (CKs) because they are known inhibitors of

root growth (Darimont et al., 1971; Scott, 1972; Svensson, 1972; Goodwin, 1978) and are thought to be synthesized in the tips of actively growing roots (Radin and Loomis, 1971; Engelbrecht, 1972; Short and Torrey, 1972; Feldman, 1975; Skene, 1975; Van Staden and Davey, 1979). In addition, CKs are exported to the shoot in the xylem, where they are assumed to be involved in the regulation of shoot development (Burrows and Carr, 1969; Alvim et al., 1976; Purse et al., 1976; Mozes and Altman, 1977; Van Staden and Davey, 1979).

There are numerous reports describing the effects of exogenously-applied CK on pine seedling development. Marcavillaca and Montaldi (1966) were able to increase fascicular bud development in three-year-old *P. elliottii* seedlings through treatment with 100 mg/l BAP for 20 days. Cohen and Shanks (1975) also induced fascicular bud formation on dwarf shoots of five-year-old *P. ponderosa* seedlings that received two foliar-spray applications, a week apart, of either 500 or 1,000 mg/l BAP. The latter was more effective than the former and stimulated even more buds when applied in conjunction with terminal bud removal. A similar response was seen by Cohen (1978) when a single treatment (foliar spray) of either 500 or 1,000 mg/l BAP was applied to three-year-old *P. strobus* seedlings. Eight weeks after treatment, fascicular buds developed on dwarf shoots along the entire stem. Several of these buds elongated, giving rise to lateral shoots. Furthermore, Whitehill and Schwabe (1975) reported the formation of intrafascicular buds along the entire length of the stems of five-year-old *P. sylvestris* seedlings in response to spraying foliage with 200 mg/l BAP, along with a wetting agent, every four to five days for one month. A similar response was seen by Kossuth (1978), who tested three levels of three CKs for bud induction on the same species.

The response to CK appears to be not only a function of species but also the mode of application. When a piece of cotton, saturated with an aqueous solution of 250 mg/l BAP, was placed in contact with the apical bud cluster of *P. densiflora* seedlings for 24 hours, a pale green cluster of buds formed at the tips of current axillary and lateral shoots. Subsequently, these buds elongated, resulting in the formation of lammas shoots (i.e., late season shoot growth that normally would remain dormant until the following spring; Odani, 1977). When an aqueous solution of 2% BAP, containing a wetting agent, was applied to one-year-old "grass-stage" *P. palustris* seedlings, two to three flushes of shoot growth occurred within a single growing season. This same treatment resulted in plants with larger buds in the fall. When higher BAP concentrations were used, primary needles proliferated at the base of the seedlings. Addition of potassium nitrate, thiourea and zinc sulfate enhanced the effect of BAP (Kossuth, 1981).

While testing various carrier solutions for their effectiveness in promoting lateral bud development on five- and six-year-old *Abies balsamea* trees, Little (1984) discovered that the BAP concentration needed to induce bud formation was beyond the solubility limit of BAP in water. To overcome this problem and minimize phytotoxicity, the final formulation contained 600 mg/l BAP, 1.5% dimethyl sulfoxide, 13.5% methanol and 0.1% Tween 20. Spraying container-grown red pine seedlings with this same carrier solution, containing 200 mg/l BAP, led to increased fascicular and terminal bud development. Despite their large terminal buds, treated seedlings did not elongate as much as control seedlings in the next growing season, although they did produce many more lateral buds. Moreover, this same treatment also led to decreases in needle and root growth and root:shoot ratios (unpublished data; see Little, 1982).

This information suggests that CKs are involved in the control of seedling development. However, before characterizing the CKs of red pine, additional evidence was needed for their involvement in the control of shoot development. It was thought that exogenous applications of CK could be used to mimic episodic growth by inducing a surge in shoot growth. Therefore, using the methodology outlined by Little (1984), a series of exogenous application experiments was undertaken. This paper reports on the results of these exogenous application trials.

MATERIALS AND METHODS

Seedling Culture

Seedlings were established and grown as described in Section I.

Identification of Carrier Components for BAP

Little (1984) used 13.5% methanol, 1.5% dimethylsulfoxide and 0.1% Tween 20 in his aqueous carrier solution used to introduce 6-benzylaminopurine (BAP) into balsam fir (*Abies balsamea*) seedlings. Various combinations of these components, in an aqueous solution, with 200 mg/l BAP (see Table 1), were applied to 13 seedlings twice weekly for three weeks. Treatments began 1.5 weeks after sowing and were applied by inverting the seedling and repeatedly submerging the foliage until all needles were completely coated with the treatment solution (hereafter referred to as the "foliar dip" method). After treatment, seedlings were returned to an upright position and were not watered for at least 12 hours.

Thirty-five weeks after sowing, half of the seedlings in each treatment group were removed from their containers. After rinsing away the potting mix with tap water, the seedlings were photographed to record their development. This process was repeated at 72 weeks.

Determining the Optimal BAP Concentration and Mode of Application

Aqueous solutions containing 0.075% (v/v) Tween 20 and various concentrations of BAP (0, 2.5, 25, 50, 100 and 200 mg/l) were applied to seedlings twice weekly for three consecutive weeks using the foliar dip method described above. Two hundred microliters (μ l) of the same concentration series was applied twice weekly to the surface of the potting mix near the base of the stems in a separate

Table 1. Composition of various treatment solutions used to test the components of a carrier solution needed to introduce BAP into red pine seedlings

Tmt. Group	Volume ^a Water ^b	Volume MeOH	Volume DMSO	Vol. BAP Stock ^c	Volume Tween 20
1	20.0				
2	17.3	2.7			
3	17.0	2.7	0.3		
4	16.6	2.7	0.3	0.4	
5	16.6	2.7	0.3	0.4	0.015
6	19.7		0.3		
7	19.3		0.3	0.4	
8	19.3		0.3	0.4	0.015
9	19.6			0.4	
10	19.6			0.4	0.015
11	20.0				0.015
12	17.3	2.7			0.015
13	19.7		0.3		0.015
14	16.9	2.7		0.4	
15	16.9	2.7		0.4	0.015

^aAll volumes are given in milliliters.

^bDeionized distilled water was used.

^cThe stock solution contained 10.0 mg/ml BAP.

group of seedlings during the same three-week interval. Treatments were begun 1.5 weeks after sowing. Each treatment group contained 17 seedlings, and there was one set of control seedlings for each mode of application, for a total of 204 seedlings.

There were no obvious differences between the control seedlings and those growing in the cells receiving soil-surface applications of BAP. Therefore, this portion of the study was repeated. This time, 500 μ l of a solution containing 0.075% Tween 20 and 200 mg/l BAP was applied to the root zone. Twenty seedlings were treated twice weekly for six weeks, whereas the control seedlings received only deionized, distilled water.

Response to BAP as Affected by Application Number and Seedling Age

This experiment was begun when seedlings were three weeks of age. Cotyledons were fully expanded and nearly all seed coats had been shed. Benzylaminopurine, at 25 mg/l, was applied in an aqueous solution of 0.075% Tween 20 by the foliar-dip method. Each treatment consisted of 10 replicate seedlings. The design was factorial; the number of successive BAP applications, 0.5 weeks apart, varied from one to six; and BAP applications were commenced at six different seedling ages: 3, 4, 5, 6, 7, and 8 weeks. Control seedlings received no BAP (only water + Tween 20).

Seed Treatment

The effects of exogenous BAP applications on newly-germinated red pine seeds were also tested. After the appearance of a radicle hook, 80 of the seeds were immersed in an aqueous solution of 200 mg/l BAP and 0.075% Tween 20. Twenty seeds were removed at each of four sampling times: 15, 30, 90, and 180

minutes. Immediately after removal from the treatment solution, the seeds were planted in Forestry Mix at field capacity in Leach Pine Cells. Twenty seeds were transferred directly from the blotter paper to the potting mix to serve as controls. Once again, the cultural practices used for the resulting seedlings are described in Section I.

RESULTS

Identification of the Carrier Components for BAP

The combined effect of BAP and Tween 20 was striking 13.7 weeks after commencing the foliar applications. When BAP and Tween 20 were applied together, the morphology of the shoot was drastically altered by the precocious development of preformed buds and by the production of adventitious buds (Figure 1A; histological evidence for adventitious buds is presented in Appendix A). Both components were required for this response; when Tween 20 or BAP were applied alone, there was no visible effect (Figure 1B). The presence of DMSO or methanol in the carrier solution also had no visible effect on red pine shoot development. Therefore, in all subsequent experiments these components were excluded, and an aqueous solution of Tween 20 was routinely used as the carrier.

Half of the seedlings from each treatment group were harvested 35 weeks after sowing to observe total seedling development. As compared to the controls, the BAP-treated seedlings had underdeveloped root systems, shorter hypocotyls, fewer primary leaves, no secondary leaves, and terminal clusters of adventitious buds (Figure 2A). Seventy-two weeks after sowing, the rest of the seedlings were harvested. Although the seedlings receiving both BAP and Tween 20 had foliar development similar to that of the controls, they still had underdeveloped root systems at the time of the final harvest (Figure 2B).

Determining the Optimal BAP Concentration and Mode of Application

Seedlings receiving various concentrations of BAP were photographed 5.1 weeks from sowing. Figures 3A through 3F represent seedlings receiving 0, 2.5, 25, 50, 100, 200 mg/l, respectively, using the foliar dip technique. As the concen-

Figure 1. The effect of BAP on red pine shoot development. Treatments began when the seedlings were 1.5 weeks old; photos were taken when they were 17.5 weeks old. The scale in both photos is the same; the bud cluster is approximately 1 cm in diameter

(A) A seedling which received biweekly treatments of 200 mg/l BAP, in an aqueous solution of 0.075% Tween 20 (the carrier), for three consecutive weeks

(B) A control seedling which received the same number of applications of the carrier solution alone

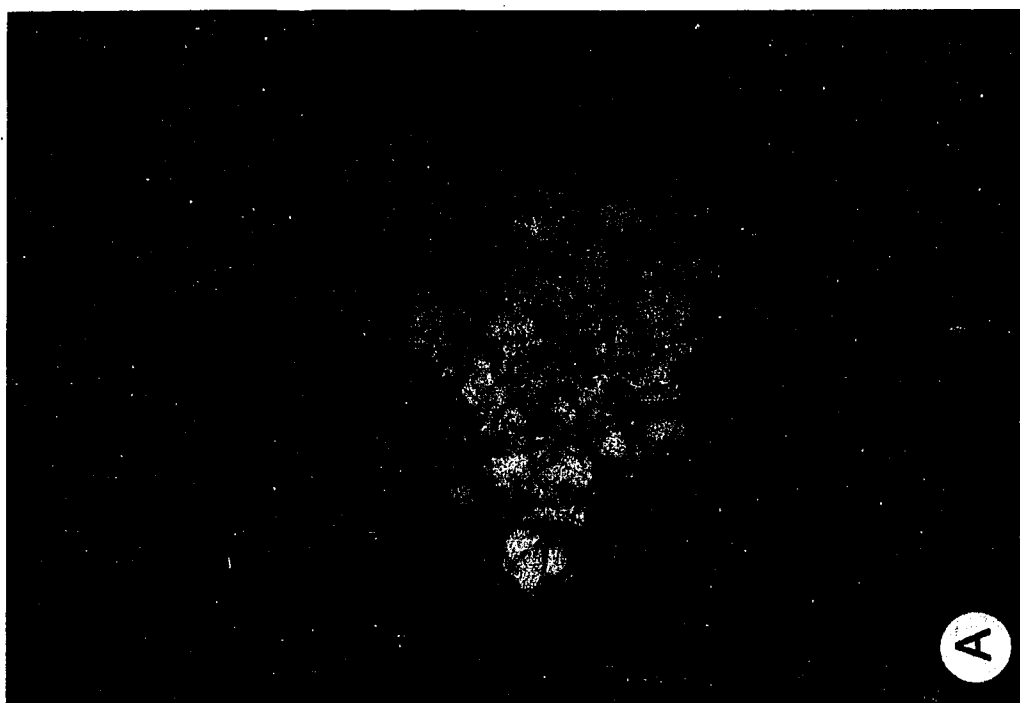
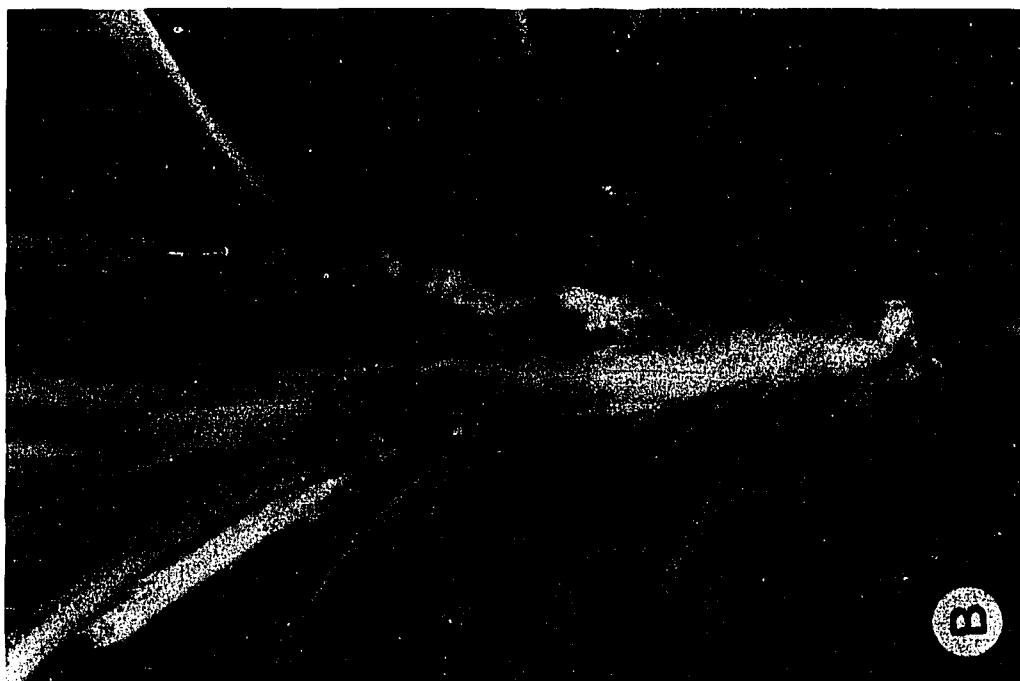


Figure 2. The long-term effect of BAP on red pine root development.
Treatments were begun when the seedlings were 1.5 weeks old. Seedlings received six biweekly treatments of either the carrier solution alone (left-hand seedling in each photo) or the carrier solution containing 200 mg/l BAP (right-hand seedling in each photo)

(A) 35-week-old seedlings. The scale is 6 in. (15.2 cm) long

(B) 72-week-old seedlings. The ruler has inches inscribed on its left-hand edge and centimeters on its right-hand edge

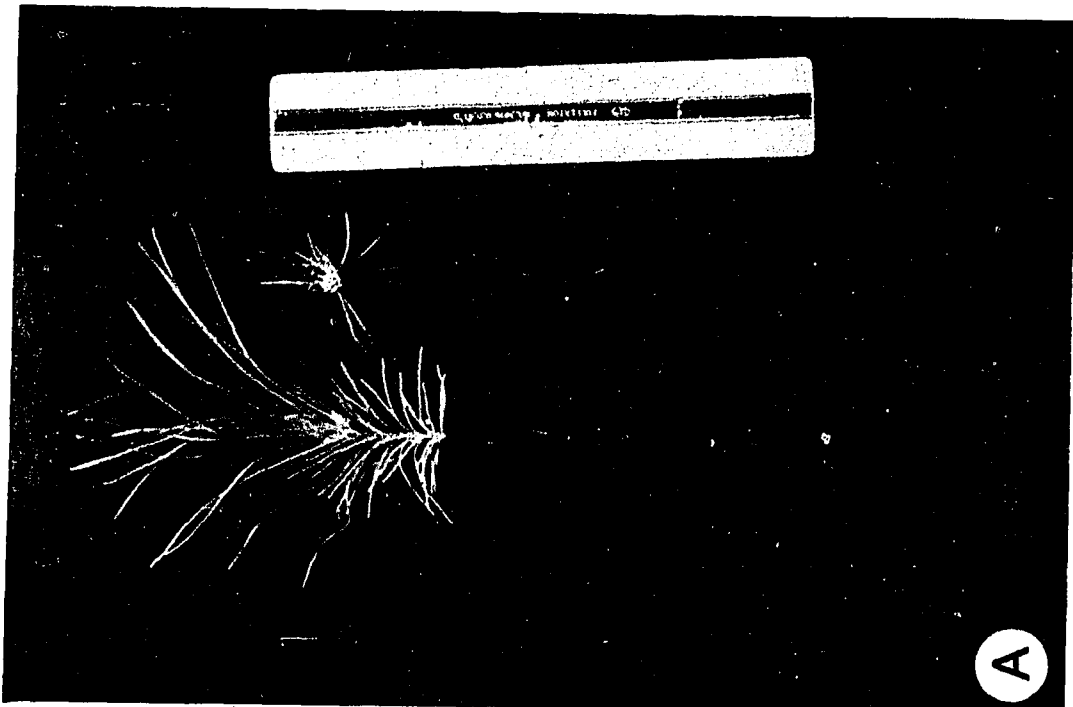
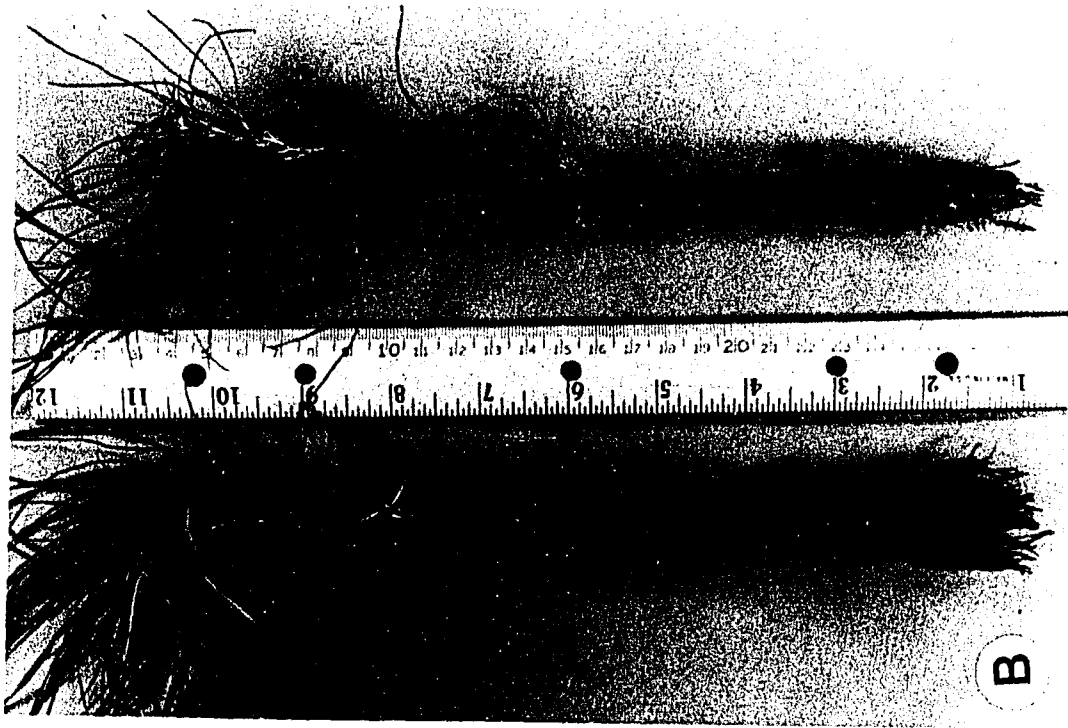
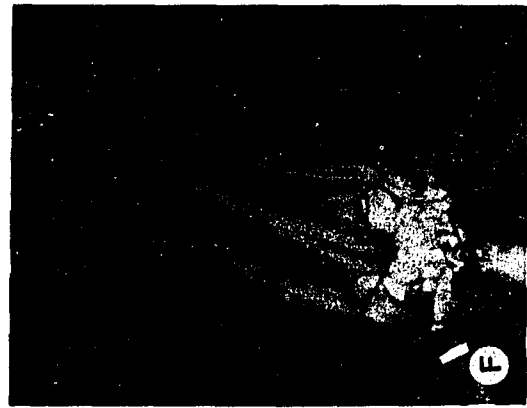
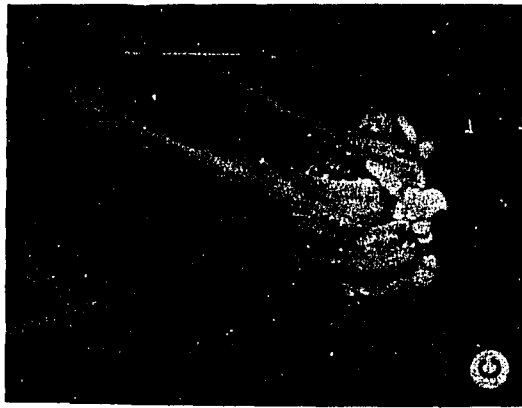


Figure 3. Seedlings representative of those receiving six biweekly treatments of the carrier solution containing various concentrations of BAP (0, 2.5, 25, 50, 100, 200 mg/l; A-F, respectively) applied directly to the foliage. Treatments were begun when the seedlings were 1.5 weeks old; photos were taken when the seedlings were about 5 weeks of age. The hypocotyls are about 2 mm in diameter



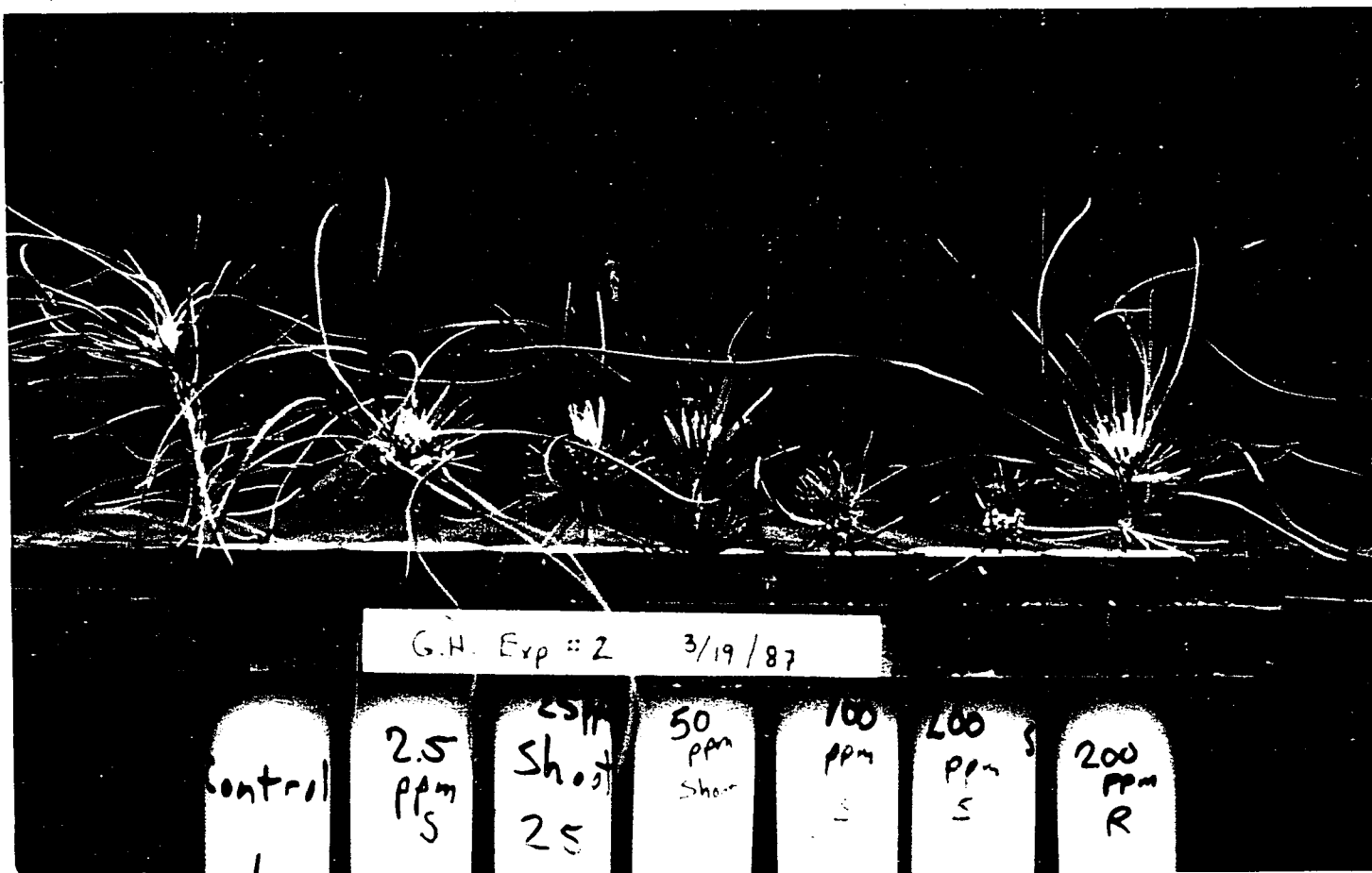
tration of BAP increased, there was a corresponding increase in the number of buds formed. Hypocotyl length was inversely proportional to the BAP concentration and the percentage of induced buds that gave rise to lateral shoots decreased as the concentration of BAP increased (Figures 3 and 4).

Although some of the control seedlings had axillary buds, there seemed to be more seedlings with buds in the group receiving 200 mg/l BAP on the surface of the potting mix than in the controls. Because of this uncertainty, the root uptake experiment was repeated. Seedlings contained within the cells receiving 500 μ l of 200 mg/l BAP on the potting mix two times per week were photographed three weeks after treatments were begun. In Figure 5 it can be seen that the treated seedlings had visible axillary buds at various positions along their stems, whereas the control seedlings had no observable axillary bud development.

Response to BAP as Affected by Application Number and Seedling Age

About 10.7 weeks after the first treatment was administered, seedlings receiving various concentrations of BAP at different ages were photographed (Figures 6A-6F). The younger the seedlings were when they received their first BAP treatment and the more applications they received, the greater the affect on hypocotyl length. A greater number of BAP treatments also induced the formation of proportionately more adventitious buds. Buds produced on seedlings treated in the first week were clustered around the cotyledons, buds produced on seedlings treated in the sixth week were predominantly located at the base of the terminal bud. Nearly all of the buds produced by the seedlings treated in the first week grew out. As the age at which seedlings received their first treatment of BAP increased, the percentage of buds that elongated decreased (Figure 6B vs.

Figure 4. Seedlings representative of those receiving six treatments of the carrier solution containing various concentrations of BAP (0, 2.5, 25, 50, 100, 200 mg/l) applied directly to the foliage and 200 µl of the carrier solution containing 200 mg/l BAP applied to the potting mix near the base of the seedling (in order from left to right). Treatments were begun when the seedlings were 1.5 weeks old; photos were taken when the seedlings were 23.5 weeks of age. All containers are 1 in. (2.5 cm) in diameter



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Control

2.5
ppm
S

50
ppm
Shoot
25

50
ppm
Shoot

100
ppm
S

200
ppm
S

200
ppm
R

Figure 5. Seedlings receiving 500 μ l of the carrier solution containing 200 mg/l BAP (three on left) and containing no BAP (three on right) twice weekly for 6 consecutive weeks, beginning when the seedlings were 1.5 weeks old. The photo was taken 3 weeks after treatments were begun

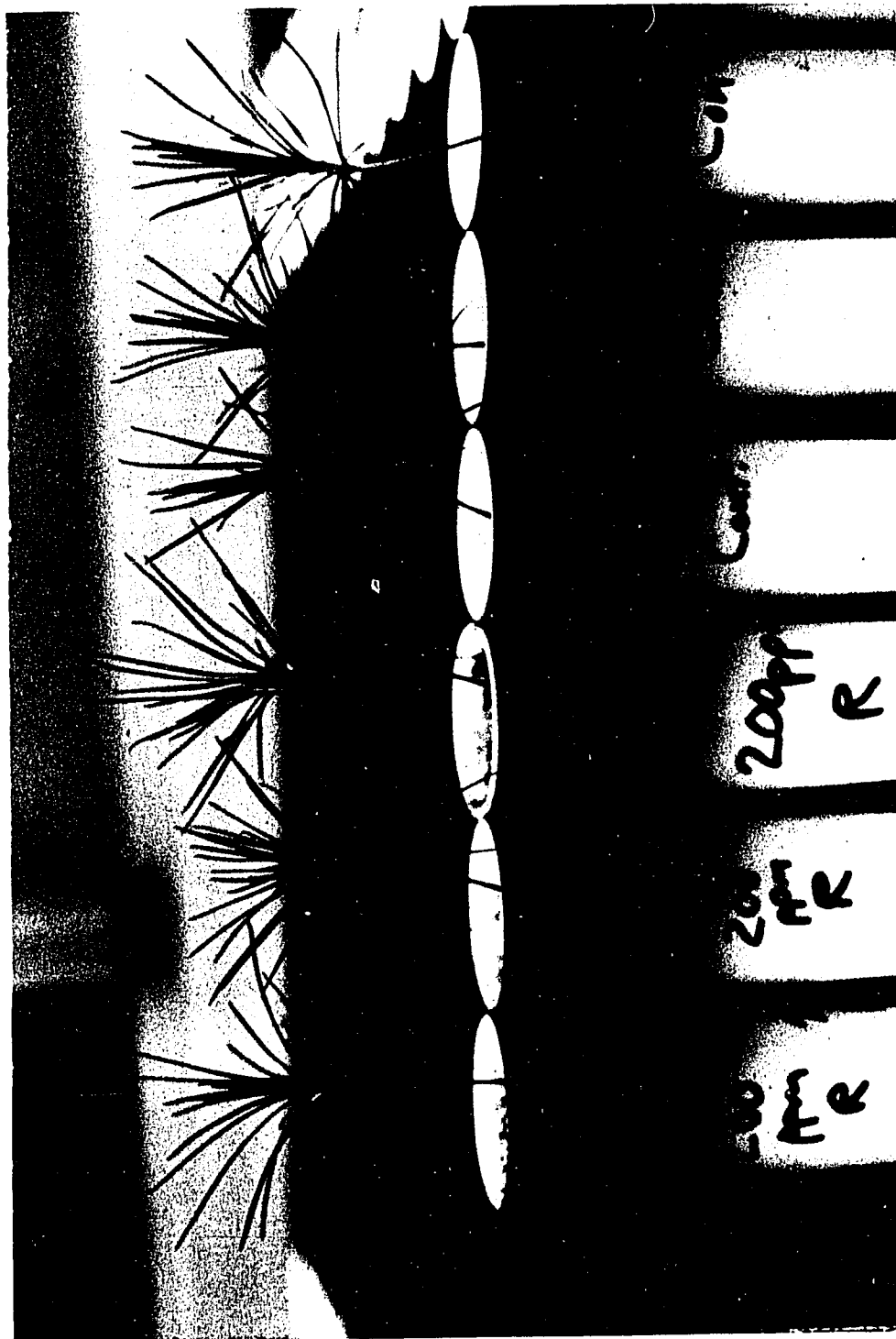


Figure 6. Seedlings which received a varying number of treatments of the carrier solution containing 25 mg/l BAP, at various ages. The first number in the two-number code (on the containers) refers to the age (in weeks) at which foliar applications were begun. The second number refers to the total number of biweekly treatments received. Control seedlings (labelled "C") received foliar applications of the carrier solution containing no BAP. Treatments were begun when the seedlings were 3 weeks old; photos were taken 10.7 weeks later

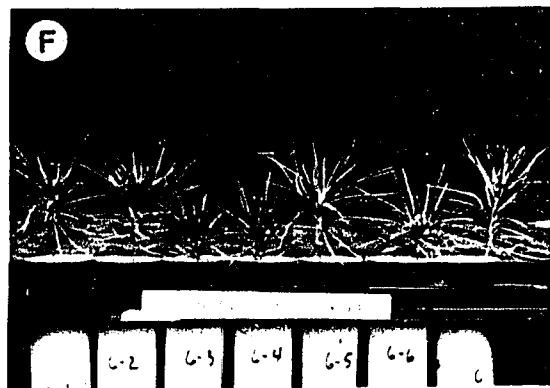
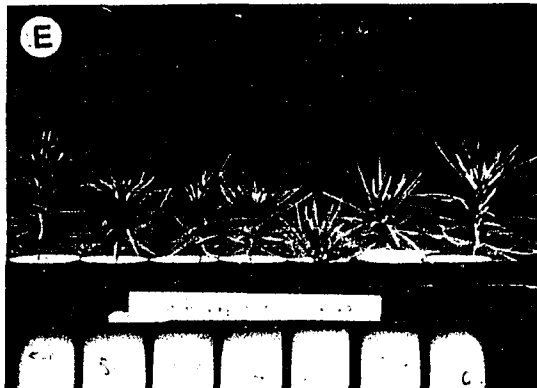
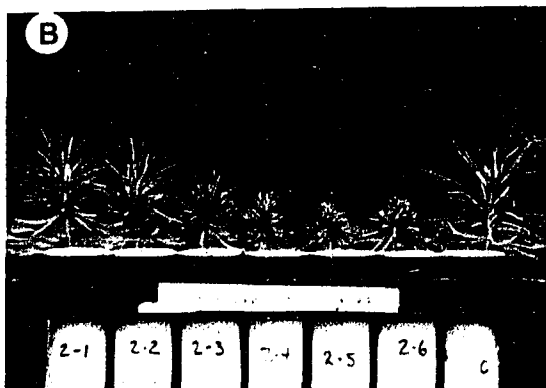
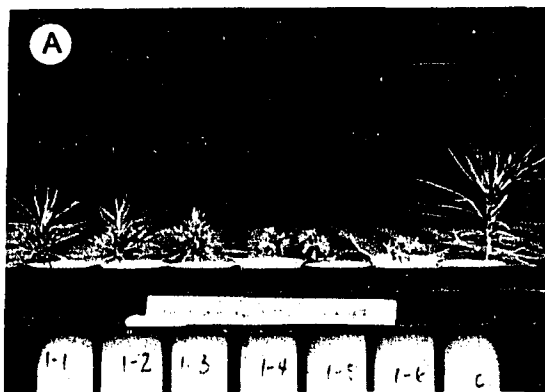


Figure 6D).

Seed Treatment

Longer exposures to BAP led to lower seed germination and seedling survival (Table 2) and commensurately shorter hypocotyls and needles (Figure 7). Seedlings receiving the longest exposure to BAP also exhibited malformed needles with necrotic tips (Figure 7).

Table 2. Percent germination and percent survival of seeds pretreated with 200 ppm BAP and 0.075% Tween 20

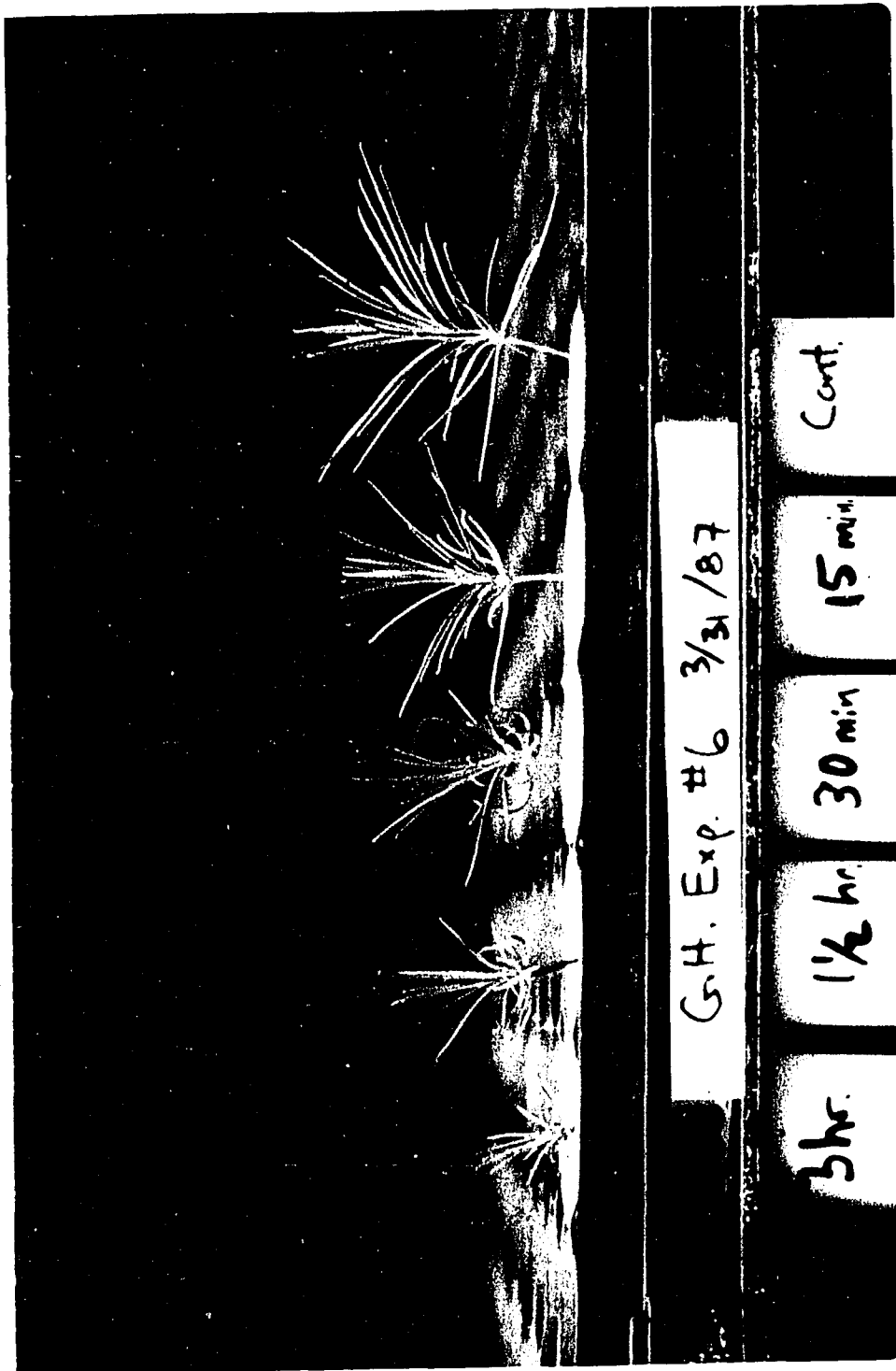
Tmt.	% Germ. ^a	% Survival ^b
control	100	100
15 min. ^c	70	70
30 min.	65	60
90 min.	50	45
180 min.	35	25

^aTaken after seed coats shed, ca. 3 wks. after tmt.

^bTaken at two months from sowing.

^cLength of treatment.

Figure 7. Seedlings resulting from germinating red pine seeds treated for various lengths of time with the carrier solution containing 200 mg/l BAP. Control seedlings received treatment with only the carrier. The photo was taken 9 weeks after treatment



G.H. Exp. #6 3/31/87

3 hr.

1 1/2 hr.

30 min.

15 min.

Cont.

DISCUSSION

The only two components of the carrier solution needed to foster bud development were the surfactant and BAP. If either of these was absent from the treatment solution, no buds were formed. The other carrier components tested had no observable affect on seedling morphology when applied singly or in combination with any of the other components. Therefore, it would appear that the cytokinin-containing solution merely needs to be kept in intimate contact with the leaf surface long enough for the cytokinin to be taken up by the leaf.

As has already been stated, cytokinins are known to inhibit root development. This effect is manifest in at least two ways. First, when applied exogenously, CKs are potent inhibitors of root elongation. This has been demonstrated repeatedly in a wide range of species (see Darimont et al., 1971; Scott, 1972; Svensson, 1972; and Goodwin, 1978 for an exhaustive list of references). Svensson (1972) illustrated that the reduction in root growth seen in maize (*Zea mays*) and wheat (*Triticum vulgare*) is caused by an inhibition of cell elongation and of transverse cell divisions. This leads to the production of short, swollen roots, as observed by Wittwer and Dedolph (1963).

The second way in which CKs affect root development is through their influence on root initiation. Cytokinins have been shown to inhibit root formation on petioles and hypocotyls of dwarf bean (*Phaseolus vulgaris*; Humphries, 1960), on pea (*Pisum sativum*) stem sections (Kaminek, 1968), on excised rye (*Secale cereale*) roots (Yang and Dodson, 1970), and on pea cuttings (Eriksen, 1974; Bollmark and Eliasson, 1986). The combined inhibition of root initiation and elongation leads to severely underdeveloped root systems.

Although the results of Yang and Dodson (1970) suggest that root inhibition is a direct result of CK application, that isn't necessarily the case in this study. Cytokinin applications could inhibit root growth by some as yet unknown direct means or by creating a stronger sink at the shoot apex (i.e., the developing bud cluster), thus diverting carbon away from the roots. The latter explanation is supported by the work of Turvey and Patrick (1979) in which kinetin promoted the transport of ^{14}C - and ^{32}P -labelled assimilates to the site of hormone application (stumps of decapitated *Phaseolus* plants). Similar results were obtained by Clark and Hackett (1979), who treated intact shoot tips of English ivy (*Hedera helix*) with BAP.

In the carrier component identification experiment, the same concentration of BAP was used for all treatments, so root development was inhibited equally in all treatments in which there was an effect. The seedlings from the experiment used to determine the optimal BAP concentration and mode of application were not harvested until very much later because they were used in another experiment. By the time they were harvested, there were no significant differences in root growth among treatments. Therefore, it is not known if the level of root inhibition was correlated with BAP concentration. Even so, it is difficult to dissociate carbohydrate diversion from direct cytokinin inhibition because higher levels of BAP induced more buds and, hence, a greater carbohydrate demand.

It is obvious that, at the higher BAP concentrations, there are more buds than leaf axils. Therefore, some of these buds must be adventitious. Moreover, because not all primary leaf axils contain buds (Lester, 1968), BAP may be inducing the development of axillary buds in addition to adventitious buds. Although more of the treated seedlings in the second root uptake experiment had axillary

buds and those with buds appeared to have more buds than the control seedlings, the latter were not completely devoid of axillary buds. Also, there were fewer buds induced by the BAP taken up through the roots, and those buds that did form were distributed along the hypocotyl rather than being clustered in a single location.

The level of shoot stunting at various ages is, once again, difficult to attribute to direct cytokinin action. If carbohydrate is being diverted away from foliar production early in seedling development, there is less photosynthetic capacity available for future shoot development once bud formation ceases. If, on the other hand, primary needles develop prior to the onset of BAP treatment, there will be more leaf area to satisfy the photosynthate demand of the newly initiated buds as well as the remainder of the developing seedling.

It is worth mentioning that the response to BAP was age dependent. Nearly all of the buds induced on the younger seedlings flushed soon after formation, whereas few of the buds induced on the older seedlings flushed. This may be due to reduced production of endogenous PGRs, to a change in sensitivity of the seedlings over time, or to a change in the resistance to absorption by the different needle types (cotyledons vs. primary needles). In addition, the enzyme system for interconverting or otherwise metabolizing the absorbed BAP may become deactivated at a certain stage in seedling development (Stuchbury and Burch, 1987). Because the internal levels of cytokinin were not monitored, it is not known how much BAP actually got into the treated tissues or whether what got into the plant was converted to another form of cytokinin or metabolized.

Until the last phase of this study (see Sections III and IV), the types of cytokinins produced by red pine and their normal endogenous levels had never been determined. This, coupled with the uncertain fate of the absorbed BAP, made it

difficult to know how much to apply exogenously. Although we did not anticipate the observed response, we are confident of our ability to alter red pine development. The next phase of this research will be to measure the levels of all native cytokinins in various tissues throughout early ontogeny. Hopefully this information will shed some light on the mechanism for controlling episodic growth and other aspects of seedling development.

Finally, it should be noted that this terminal cluster of adventitious buds could possibly serve as a means of micropropagating red pine. We have already developed a technique for sterilizing the buds and have identified the proportions of exogenous auxin and cytokinin in an agar tissue culture medium needed to induce some of these buds to flush. Ultimately, we would like to separate the buds that have flushed from the cluster and root them (see Appendix B).

LITERATURE CITED

- Alvim, R., E. W. Hewett and P. F. Saunders. 1976. Seasonal variation in the hormone content of willow. I. Changes in abscisic acid content and cytokinin activity in the xylem sap. *Plant Physiology* 57: 474-476.
- Bollmark, M. and L. Eliasson. 1986. Effects of exogenous cytokinins on root formation in pea cuttings. *Physiologia Plantarum* 68: 662-666.
- Burrows, W. J. and D. J. Carr. 1969. Effects of flooding the root system of sunflower plants on the cytokinin content in xylem sap. *Physiologia Plantarum* 22: 1105-1112.
- Clark, J. R. and W. P. Hackett. 1979. Distribution of soluble ^{14}C -labelled assimilates as related to distribution of ^{14}C -benzyladenine in shoot tips of *Hedera helix*. *Physiologia Plantarum* 47: 87-90.
- Clements, J. R. 1970. Shoot responses of young red pine to watering applied over two seasons. *Canadian Journal of Botany* 48: 75-80.
- Cohen, M. A. 1978. Shoot apex development and rooting of *Pinus strobus* L. by dwarf shoots. *Journal of the American Society for Horticultural Science* 103(4): 483-484.
- Cohen, M. A. and J. Shanks. 1975. Effect of $\text{N}^6\text{-BA}$, GA_3 , and removal of terminal buds on dwarf shoot development in *Pinus ponderosa*. *Journal of the American Society for Horticultural Science* 100(4): 404-406.
- Darimont, E., T. Gaspar, and M. Hofinger. 1971. Auxin-kinetin interaction on the lentil root growth in relation to indoleacrylic acid metabolism. *Zeitschrift für Pflanzenphysiologie* 64: 232-240.
- Doak, C. C. 1935. Evolution of foliar types, dwarf shoots, and cone scales of *Pinus*. *Illinois Biological Monographs* 13: 1-106.
- Drew, A. P. 1982. Shoot-root plasticity and episodic growth in red pine seedlings. *Annals of Botany* 49: 347-357.
- Drew, A. P. and F. T. Ledig. 1980. Episodic growth and relative shoot:root balance in loblolly pine seedlings. *Annals of Botany* 45: 143-148.
- Duff, G. H. and N. J. Nolan. 1958. Growth and morphogenesis in the Canadian forest species. III. The time scale of morphogenesis at the stem apex in *Pinus resinosa* Ait. *Canadian Journal of Botany* 36: 687-706.

- Eggleston, K. L. and R. C. Sharp. 1986. Fertilizer trials on containerized red pine. Pages 38-42 in T. D. Landis and J. W. Fischer, eds. Proceedings of the Intermountain Nurseryman's Association Meeting, Fort Collins, CO, August 13-15, 1985. USDA Forest Service General Technical Report RM-125. 111 pp.
- Engelbrecht, L. 1972. Cytokinins in leaf cuttings of *Phaseolus vulgaris* L. during their development. *Biochemie und Physiologie der Pflanzen* 169: 317-320.
- Eriksen, E. N. 1974. Root formation in pea cuttings. III. The influence of cytokinin at different developmental stages. *Physiologia Plantarum* 30: 163-167.
- Feldman, L. J. 1975. Cytokinins and quiescent centre activity in roots of *Zea*. Pages 55-72 in J. G. Torrey and D. T. Clarkson, eds. The development and function of roots. Academic Press, London.
- Glerum, C. and G. Pierpoint. 1968. The influence of soil moisture deficits on seedling growth of three coniferous species. *The Forestry Chronicle* 44: 26-29.
- Goodwin, P. B. 1978. Phytohormones and growth and development of organs of the vegetative plant. Pages 31-173 in D. S. Letham, P. B. Goodwin and T. J. V. Higgins, eds. Phytohormones and related compounds: A comprehensive treatise. Vol. II. Elsevier/North-Holland Biomedical Press, The Netherlands.
- Humphries, E. C. 1960. Inhibition of root development on petioles and hypocotyls of dwarf bean (*Phaseolus vulgaris*) by kinetin. *Physiologia Plantarum* 13: 659-663.
- Kaminek, M. 1968. Dynamics of amino acids in pea stem sections during root formation and its inhibition by kinetin and ethionine. *Biologia Plantarum* 10(6): 462-471.
- Kossuth, S. V. 1978. Induction of fascicular bud development in *Pinus sylvestris* L. *HortScience* 13(2): 174-176.
- Kossuth, S. V. 1981. Shortening the grass stage of longleaf pine with plant growth regulators. *Forest Science* 27(2): 400-404.
- Ledig, T. T., A. P. Drew, and J. G. Clark. 1976. Maintenance and constructive respiration, photosynthesis, and net assimilation rate in seedlings of pitch pine (*Pinus rigida* Mill.). *Annals of Botany* 40: 289-300.
- Lester, D. T. 1968. Developmental patterns of axillary meristematic activity in seedlings of *Pinus*. *Botanical Gazette* 129(3): 206-210.

- Little, C. H. A. 1982. Uses of plant growth regulators in the conifer nursery. Pages 25-36 in D. M. Levy, P. D. Neily, and R. D. Hallett, eds. Proceedings of the northeastern area nurseryman's conference, Halifax, Nova Scotia, July 25-29, 1982.
- Little, C. H. A. 1984. Promoting bud development in balsam fir Christmas trees with 6-benzylaminopurine. *Canadian Journal of Forest Research* 14: 447-451.
- Marcavillaca, M. C. and E. R. Montaldi. 1966. Efecto de la N⁶-benciladenina sobre el crecimiento de los fasciculos de *Pinus elliottii* Engelm (in Spanish, English summary). *Informativo de Investigaciones Agrícolas Suplemento Forestal* 17: 49-53.
- Mozes, R. and A. Altman. 1977. Characteristics of root-to-shoot transport of cytokinin 6-benzylaminopurine in intact seedlings of *Citrus aurantium*. *Physiologia Plantarum* 39: 225-232.
- Odani, K. 1977. Lammas shoot growth of *Pinus densiflora* Sieb. et Zucc. mediated by the exogenous cytokinin. *Japanese Journal of Forest Science* 59(1): 22-23.
- Paterson, J. M. 1987. Red pine needle length in relation to field performance. Pages 85-91 in T. Marty, ed. Proceedings of the Northeast Area Nurseryman's Conference, Hayward State Nursery, Hayward, WI, August 17-20, 1987.
- Paterson, J. M. and D. C. F. Fayle. 1984. Early prediction of plantation performance for red pine. *The Forestry Chronicle* 60(6): 340-344.
- Purse, J. G., R. Horgan, J. M. Horgan, and P. F. Wareing. 1976. Cytokinins in sycamore sap. *Planta* 132: 1-8.
- Radin, J. W. and R. Loomis. 1971. Changes in the cytokinin of radish roots during maturation. *Physiologia Plantarum* 25: 240-244.
- Scott, T. K. 1972. Auxins and roots. *Annual Review of Plant Physiology* 23: 235-258.
- Short, K. C. and J. G. Torrey. 1972. Cytokinins in seedling roots of pea. *Plant Physiology* 49: 155-160.
- Skene, K. G. M. 1975. Cytokinin production by roots as a factor in the control of plant growth. Pages 365-396 in J. G. Torrey and D. T. Clarkson, eds. *The development and function of roots*. Academic Press, London.

- Strothmann, R. O. 1967. The influence of light and moisture on the growth of red pine seedlings in Minnesota. *Forest Science* 13: 182-191.
- Stuchbury, T. and L. R. Burch. 1987. Enzymology of cytokinin and purine metabolism. Pages 19-34 in B. Jeffcoat and R. Horgan, eds. *Cytokinins: Plant hormones in search of a role*. British Plant Growth Regulator Group Monograph No. 14.
- Sucoff, E. 1971. Timing and rate of bud formation in *Pinus resinosa*. *Canadian Journal of Botany* 49: 1821-1832.
- Svensson, S.-B. 1972. A comparative study of the changes in root growth induced by coumarin, auxin, ethylene, kinetin and gibberellic acid. *Physiologia Plantarum* 26: 115-135.
- Turvey, P. M. and J. W. Patrick. 1979. Kinetin-promoted transport of assimilates in stems of *Phaseolus vulgaris* L. *Planta* 147: 151-155.
- Van Staden, J. and J. E. Davey. 1979. The synthesis, transport and metabolism of endogenous cytokinins. *Plant, Cell and Environment* 2: 93-106.
- Whitehill, S. J. and W. W. Schwabe. 1975. Vegetative propagation of *Pinus sylvestris*. *Physiologia Plantarum* 35: 66-71.
- Wittwer, S. H. and R. R. Dedolph. 1963. Some effects of kinetin on the growth and flowering of intact green plants. *American Journal of Botany* 50(4): 330-336.
- Yang, D.-p. and E. O. Dodson. 1970. The effect of kinetin on the growth of diploid and autotetraploid root tips of rye in vitro. *Canadian Journal of Botany* 48: 19-25.

**SECTION III. IDENTIFICATION OF THE CYTOKININS IN RED PINE
SEEDLINGS. I. THE SHOOTS**

SUMMARY

Zeatin 9-riboside has been identified in shoots of *Pinus resinosa* by GC-MS analysis of its permethyl derivative and by its retention times on Sephadex LH-20 and Spherisorb ODS-2. Based on retention times and susceptibility to enzymatic degradation, several other cytokinins have been tentatively identified. These include dihydrozeatin O-glucoside and zeatin in the basic fraction and zeatin 9-riboside monophosphate, isopentenyladenosine monophosphate (iPMP) and two glucosyl phosphate derivatives in the acidic fraction. The presence of iPMP suggests that cytokinin biosynthesis is occurring in red pine shoots.

INTRODUCTION

Red pine seedlings exhibit episodic growth. In other words, they alternate between root and shoot growth in a cyclic manner. This was originally demonstrated by Drew (1982) and was recently confirmed (see Section I). Although no attempt has ever been made to correlate endogenous levels of cytokinin (CK) and auxin to surges in root and shoot growth, it is thought that phytohormones play a role in regulating episodic growth (Drew 1982).

Because of our interest in tree seedling root development (Schultz and Thompson 1990) and because exogenous CKs are known inhibitors of root growth (Scott 1972; Eriksen 1974; Stenlid 1982; Bollmark and Eliasson 1986) and are thought to be synthesized in actively growing root tips (Radin and Loomis 1971; Engelbrecht 1972; Short and Torrey 1972; Feldman 1975; Skene 1975; Van Staden and Davey 1979), it was decided to begin by assessing the role CKs might play in the control of seedling root development. However, because CKs are exported to the shoot via the xylem, where they are assumed to be involved in the regulation of shoot development (Burrows and Carr 1969; Alvim et al. 1976; Purse et al. 1976; Mozes and Altman 1977), they may also be involved in the control of episodic growth.

It has been shown that exogenous applications of a cytokinin, benzylaminopurine, alters red pine seedling development (Meilan et al. 1987). To establish a relationship between seedling CK status and episodic growth, endogenous CKs must be quantified during several cycles of episodic growth. Due to the varying pattern of CK metabolism in different plant tissues (Palmer et al. 1981a; Scott and Horgan 1984), it is necessary to assess CK status in roots and shoots independently. Before characterizing endogenous CKs quantitatively, though, they

must first be characterized qualitatively. This paper reports on the CKs identified in the shoots of red pine seedlings.

MATERIALS AND METHODS

Source of Labelled Cytokinins

[^3H]-dihydrozeatin (DHZ; 14.4 Ci mmol $^{-1}$) was obtained from New England Nuclear (Du Pont Company, BRML-Chandler Mill, Wilmington, DE 19898). [^2H]-adenosine 5'-monophosphate (AMP; 20 Ci mmol $^{-1}$) was obtained from Amersham, U.K.

Plant Material Preparation

The roots of six-month-old red pine (*Pinus resinosa* Ait.) seedlings, grown in Terra-lite Forestry Mix (a 1:1 commercially-prepared mixture of peat and vermiculite pH-adjusted for conifer growth, W.R. Grace Co., Cambridge, MA, U.S.A., 18051) in styroblocks (Silvaseed Co., Roy, Washington, U.S.A., 98580) with four-cubic-inch (65.5 cm 3) cavities, were rinsed free of potting mix with tap water and severed at the root collar with a single-edge razor. The shoots and roots were immediately frozen in liquid nitrogen and stored at -70 $^{\circ}$ C until they were freeze-dried. After freeze-drying, the shoot tissue was ground in a Wiley Mill (20 mesh screen) and stored *in vacuo* over phosphorus pentoxide for two weeks, after which the material was sealed with Parafilm in glass jars and stored at room temperature. Fresh and dry weights (FWs and DWs) were measured on 5% of the harvest to determine moisture content (see Appendix C).

Chromatography

A column (78 cm X 2.5 cm) of Sephadex LH-20 (bead size 25-100 μm , Pharmacia LKB Biotechnology, Inc., 800 Centennial Ave., Piscataway, NJ, 08854) was continually eluted with 35% ethanol (EtOH) at 30 cm 3 h $^{-1}$. Samples were intro-

duced via a septum-sealed injector port, and 35 30-cm³ fractions were collected during each run. The column was washed with at least 1 l of 35% EtOH between each run.

The equipment and chromatographic systems used in high-performance liquid chromatography (HPLC) have been described in detail by Horgan and Kramers (1979). An analytical (150 mm X 4.5 mm) column of Spherisorb ODS-2 (Jones Chromatography USA, Inc., 8100 W. Hoover Place, Littleton, Co, 80123) reversed-phase packing material was used, and samples were eluted either isocratically or with a linear gradient of acetonitrile (CH₃CN) in water, adjusted to pH 7.0 with triethylammonium bicarbonate (TEAB), at a flow rate of 2.0 cm³ min⁻¹ (see figure legends for details). Fractions were collected at one-minute intervals and monitored using UV absorption (254 nm for underivatized compounds and 276 nm for permethylated compounds). The column was washed with CH₃CN between runs, and equilibrated in the starting concentration of eluant before each run. Occasionally the column was washed with MeOH before receiving the CH₃CN wash. Standards were always run immediately after the samples.

Extraction, Purification and Identification

Approximately 14.7 g DW finely ground and lyophilized shoot material (equivalent to 115 g FW; see Appendix C) was extracted in Bielecki solvent I (50 cm³/g DW) for 18 h at 20° C to inhibit endogenous phosphatase activity (Bielecki 1964). To this solvent, 1.28 X 10⁶ dpm [³H]-DHZ was added. The tissue was removed by filtration with Whatman no. 1 paper and resuspended in the same volume of Bielecki solvent II and stirred for 4 h at 4° C. After filtration, the extraction solvents I and II were combined and concentrated *in vacuo* using rotary film evaporation (RFE) at 35° C to a final volume of ca. 100 cm³. This aqueous

portion of the extract was centrifuged at 7,600 g for 30 min, adjusted to pH 3.0 and percolated through a cellulose phosphate column (NH₄ form, pH 3.0, 200 cm³ bed volume; Anspec Corporation, P.O. Box 7730, Ann Arbor, MI, 48107). The column was rinsed with five volumes of distilled water (dH₂O) adjusted to pH 3.0 with glacial acetic acid (acidic fraction) followed by an equal volume of 2 M ammonium hydroxide (NH₄OH; basic fraction).

The dH₂O eluate was percolated through a second cellulose phosphate column (200 cm³ bed volume), which was then rinsed with one column volume of dH₂O (pH 3.0), followed by five column volumes of base. The dH₂O eluates from both cellulose phosphate columns were combined, evaporated to dryness and stored at -20° C. The combined basic fractions were reduced in volume to ca. 100 cm³, centrifuged at 7,600 g for 30 min, adjusted to pH 8.2 and partitioned five times against dH₂O-saturated butan-1-ol (BuOH). The combined BuOH phases were reduced to dryness, dissolved in 5 cm³ of 35% ethanol, centrifuged at 11,600 g for 20 min and applied to an LH-20 column, which was eluted with 1,050 cm³ EtOH. A 250 µl aliquot from each fraction was mixed with 5 cm³ Ecoscint scintillation cocktail and counted in a Beckman (model LS-1800) liquid scintillation counter.

Half of each LH-20 fraction was freeze-dried in a Speed Vac (Savant Instruments, 110-103 Bi-county Blvd., Farmingdale, NY, 11735) and tested for CK activity using the *Amaranthus* betacyanin assay (Biddington and Thomas 1973; see Appendix D). The other half of each fraction was stored at -20° C until the bioassay was completed. Based on the bioassay results and known retention times of CKs on LH-20, the remaining portions of LH-20 fractions 11-13 were combined and freeze-dried, as were fractions 14-16 and 17-19. The residue was dissolved in 1 cm³ 5% CH₃CN in dH₂O, pH 7.0 (TEAB), and filtered centrifugally through

glass microfibre (Whatman GF/F) before HPLC purification. The fractions collected were bioassayed in their entirety.

To confirm the results of the 115-g-FW extract and to further characterize the putative CK glucoside, the entire extraction and purification procedure was repeated using the basic fraction of a 23-g-DW (equivalent to 180 g FW) shoot extract. This time, the LH-20 fractions which previously showed biological activity in the region corresponding to the retention time of CK glucosides were not bioassayed. Instead, these fractions were combined, freeze-dried and treated with β -glucosidase (from almonds; Sigma Chemical Company, P.O. Box 14598, St. Louis, MO, 63178). The residue was dissolved in 7.5 cm³ of 0.1 M sodium citrate buffer (pH 5.0) with four units β -glucosidase and incubated overnight at 37° C. This reaction mixture was loaded on a C-18 Sep-pak cartridge, which was prepared as recommended by the manufacturer (Millipore, Waters Chromatography Division, 34 Maple Street, Milford, MA, 01757) and washed with 20 cm³ dH₂O before the CKs were eluted with 5 cm³ MeOH. The methanolic eluate was dried under a stream of nitrogen at 55° C, and the residue was purified using reversed-phase HPLC. Each HPLC fraction was freeze-dried and bioassayed.

Acidic fractions from the set of second cellulose phosphate columns used to purify several shoot extracts (equivalent to 650 g FW) were combined and reduced to dryness. The residue was dissolved in 30 cm³ of dH₂O, along with 9.8 X 10⁵ dpm [2-³H]-AMP, adjusted to pH 8.0 and run through a Dowex 1-X8 column (50-100 mesh, formate form, 55 cm³ bed volume). After rinsing with five column volumes of dH₂O, the column was eluted with an equal volume of 5 M formic acid in 50% MeOH. The formic acid/MeOH rinse was reduced to dryness, dissolved in dH₂O, adjusted to pH 3.0 and run through an insoluble polyvinylpyrrolidone (PVP) column (125 cm³ bed volume, equilibrated at pH 3.5). The PVP column

was washed with five column volumes of dH₂O adjusted to pH 3.5 (Andersen and Sowers 1968), which was reduced to dryness by RFE.

The residue was dissolved in 30 cm³ 0.2 M sodium acetate buffer (pH 4.8) containing 0.1% bovine serum albumin (BSA, w/v) and allowed to stand at room temperature for 3 h before two units of acid phosphatase (from wheat germ, Sigma Chemical Company) were added and the mixture was incubated overnight at 37° C. The reaction mixture was then adjusted to pH 3.0 and percolated through a cellulose phosphate column (40 cm³ bed volume), which was rinsed with five column volumes each of dH₂O (pH 3.0) and 2 M NH₄OH. The acidic fraction from this column was dried down, dissolved in buffer and again treated with acid phosphatase and purified on the reequilibrated cellulose phosphate column. This process was repeated until most of the radioactivity eluted from the cellulose phosphate column in the basic fraction (as [2-³H]-adenosine).

The basic fractions from all of the cellulose phosphate columns used to purify the acid phosphatase reaction mixtures were then combined and purified in the same way as the combined basic fractions from the cellulose phosphate columns used to purify the original extract (i.e., reduced in volume; adjusted to pH 8.2; partitioned against BuOH; dried; dissolved in 35% EtOH; centrifuged; chromatographed on LH-20; treated with β -glucosidase, if necessary; chromatographed on ODS-2; and bioassayed). LH-20 fractions 11-13, 14-16 and 22-24 were combined and purified further. These corresponded to the retention times of CK glucosides, CK ribosides and isopentenyladenosine (iPA), respectively (Horgan and Scott 1987). All of the HPLC fractions from the run used to purify the residue of LH-20 fractions 11-13 were bioassayed but only the fractions bracketing the retention times of zeatin 9-riboside (ZR) and iPA from the HPLC runs used to purify the residue of LH-20 fractions 14-16 and 22-24 were bioassayed.

In an attempt to obtain mass spectra for the tentatively identified cytokinins, the entire purification procedure was repeated with the basic fraction of a 73-g-DW (equivalent to 570 g FW) shoot extract. Once again, the LH-20 fractions were not bioassayed. Because the evidence for the glucoside from the last extract were inconclusive, the entire residue from the LH-20 fractions 11-13 was treated with β -glucosidase, chromatographed on ODS-2 and bioassayed. The residues of all other LH-20 fractions which had previously shown biological activity were chromatographed on ODS-2. The HPLC fraction residues were dissolved in MeOH and a portion of this was bioassayed, to confirm the presence of biological activity; the remainder was permethylated (Scott et al. 1980) and run, isocratically, on an ODS-2 column (see figure legend for conditions). Fractions that co-chromatographed with the appropriate permethylated standards were collected, dried and analyzed via gas chromatography-mass spectrometry (GC-MS) as described by Palmer et al. (1981b).

RESULTS

Basic Fraction

Three prominent peaks of biological activity were detected in the fractions from the LH-20 column used to purify the basic fraction of the 14.7-g-DW shoot extract (Fig. 1). The bioassay of the fractions from the HPLC column used to purify the combined LH-20 fractions 11-13 revealed a single peak of biological activity, which co-chromatographed with a dihydrozeatin O-glucoside (DHZOG) standard (Fig. 2). When LH-20 fractions 11-13 from the basic fraction of the 23-g-DW shoot extract were treated with β -glucosidase prior to HPLC purification, two peaks of biological activity were observed. One peak was centered on the retention time of DHZOG, the other on the retention time of DHZ (Fig. 3). Analytical HPLC and subsequent bioassay of the residues from the combined LH-20 fractions 14-16 and 17-19 indicated that compounds co-chromatographing with ZR and zeatin (Z) were responsible for the biological activity (Figs. 4 and 5). Similar results were obtained from both the 14.7- and 23-g-DW extracts.

Acidic Fraction

Two peaks of biological activity were detected in the bioassay of the HPLC fractions from the run used to purify the residue of LH-20 fractions 11-13. One peak was present between the retention times of zeatin O-glucoside (ZOG) and zeatin 9-glucoside (Z9G), the other was centered on the retention time of ZR (Fig. 6). Bioassay of the HPLC fractions from the runs used to purify the residue of LH-20 fractions 14-16 and 22-24 revealed biological activity co-chromatographing with ZR and iPA standards, respectively (Fig. 7).

Figure 1. *Amaranthus* betacyanin bioassay profile of the basic fraction from the 115-g-FW extract following LH-20 chromatography. The horizontal bars indicate the regions within which known standards elute. These are as follows: 1, CK glucosides; 2, CK ribosides; and 3, CK free bases. Asterisks indicate fractions containing [³H]-DHZ; the number of asterisks is proportional to the activity present. Fractions 11-13 were combined for further purification, as were 14-16 and 17-19

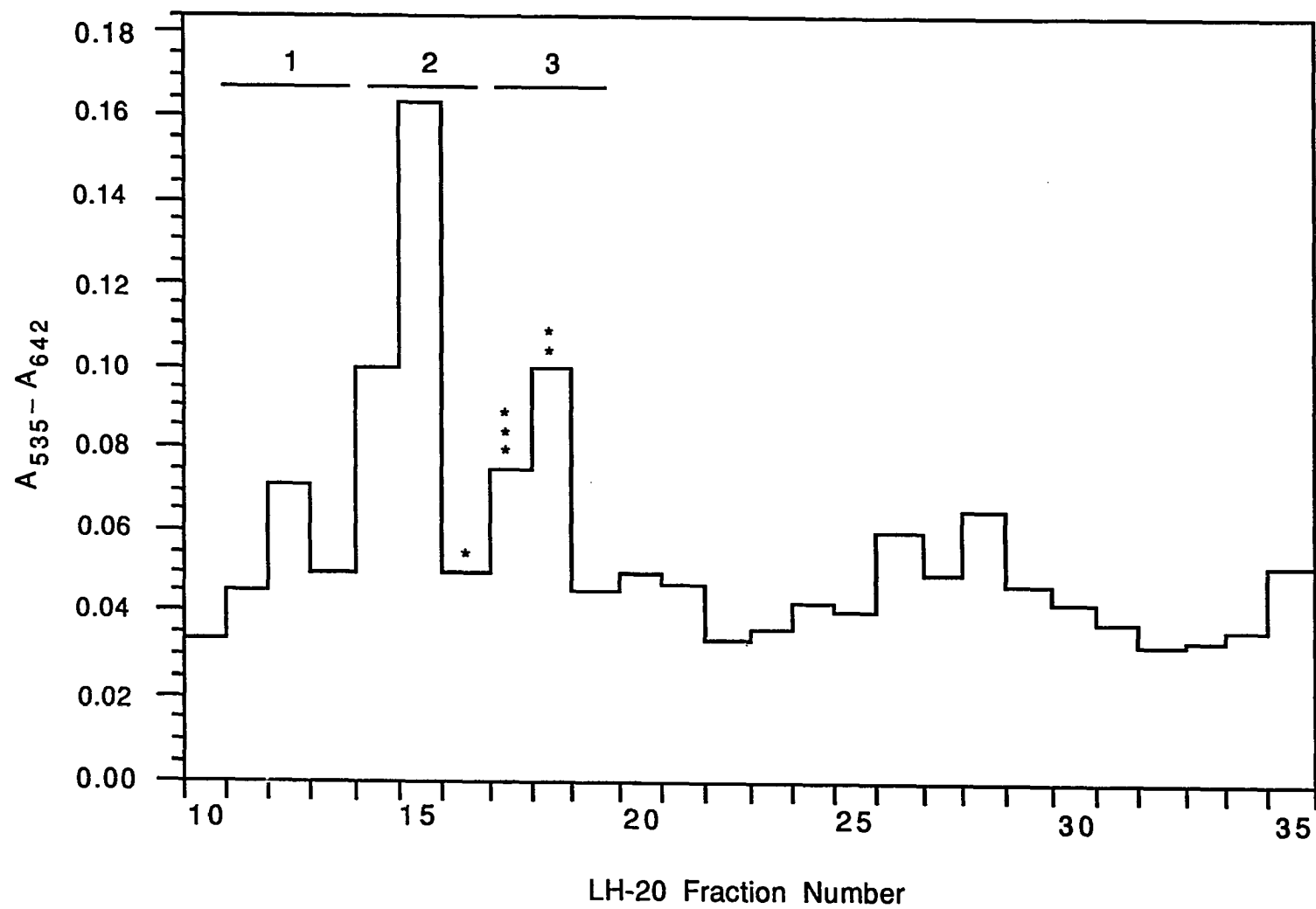


Figure 2. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 11-13 following HPLC purification. Unless otherwise indicated a 150 mm X 4.5 mm column of ODS-2 was eluted with H₂O containing a linearly increasing concentration of acetonitrile (5-20% over 40 min.) at a flow rate of 2.0 cm³/min. Fractions were collected at one-minute intervals. The retention time of DHZOG (and Z) is designated by the horizontal bar labelled "1"

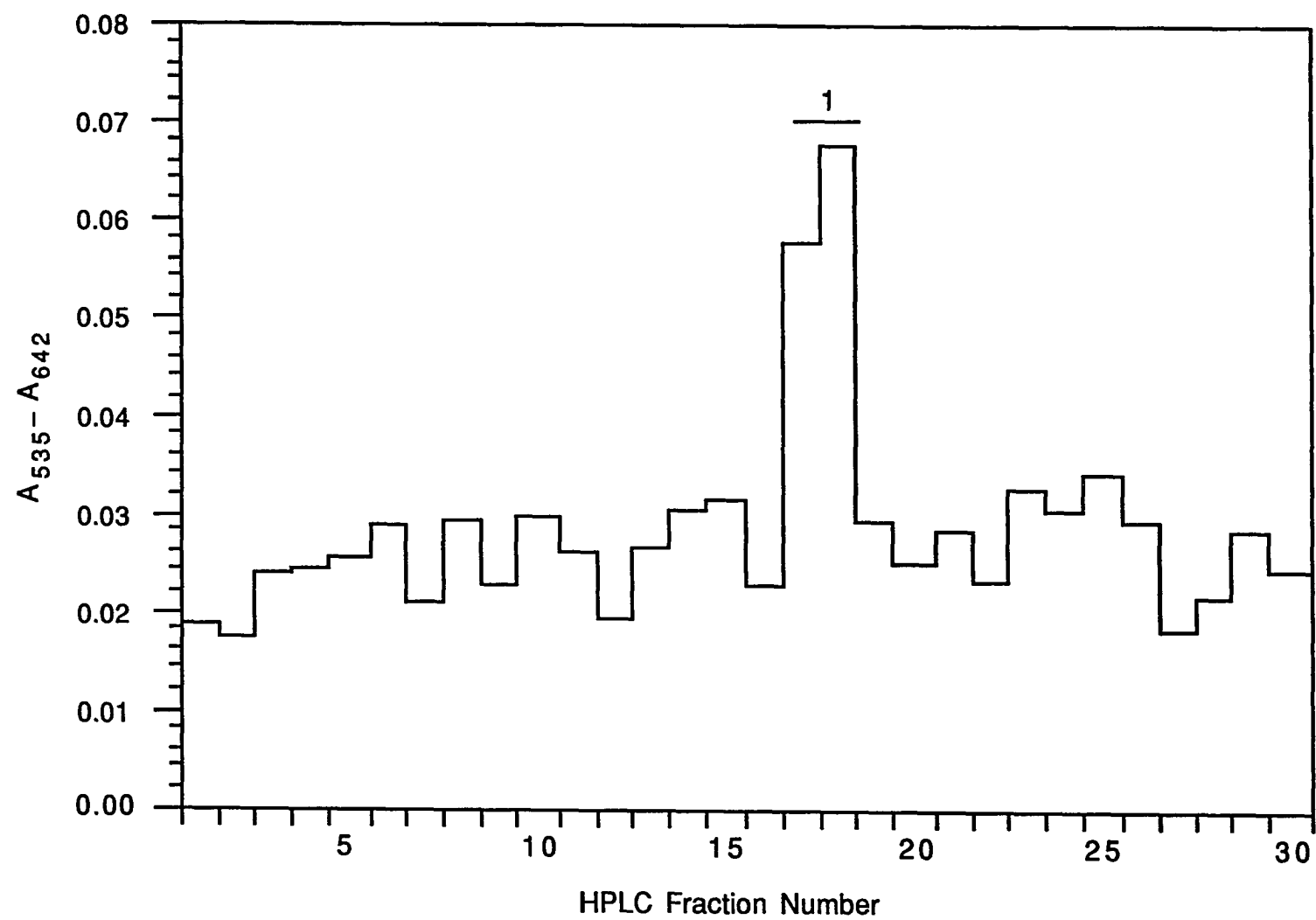


Figure 3. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 11-13 after treatment with β -glucosidase and HPLC purification. Known standards eluted in the following regions: 1, ZOG; 2, DHZOG; and 3, DHZ

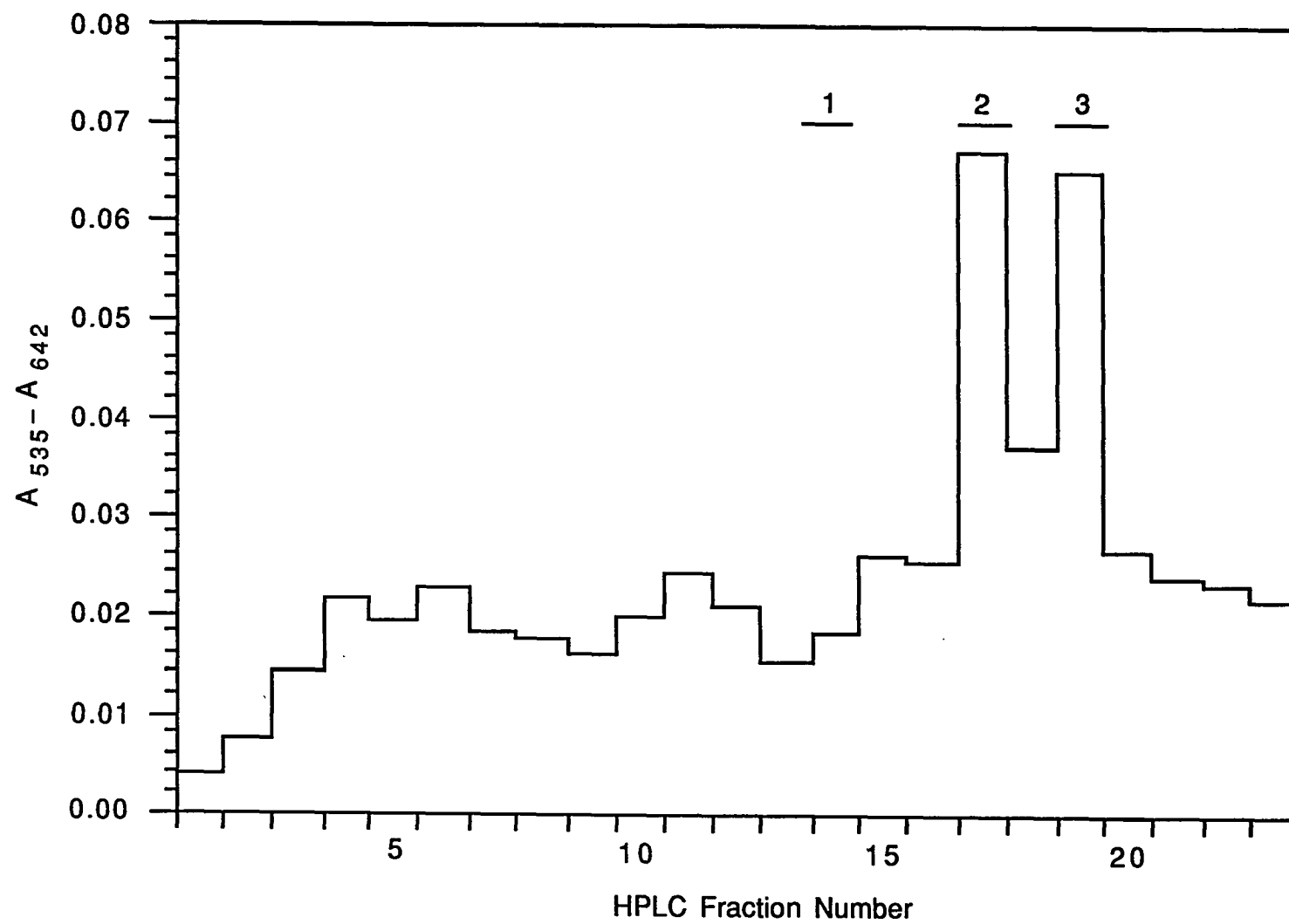


Figure 4. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 14-16 following HPLC purification. Known standards eluted in the following regions: 1, DHZOG/Z and 2, ZR

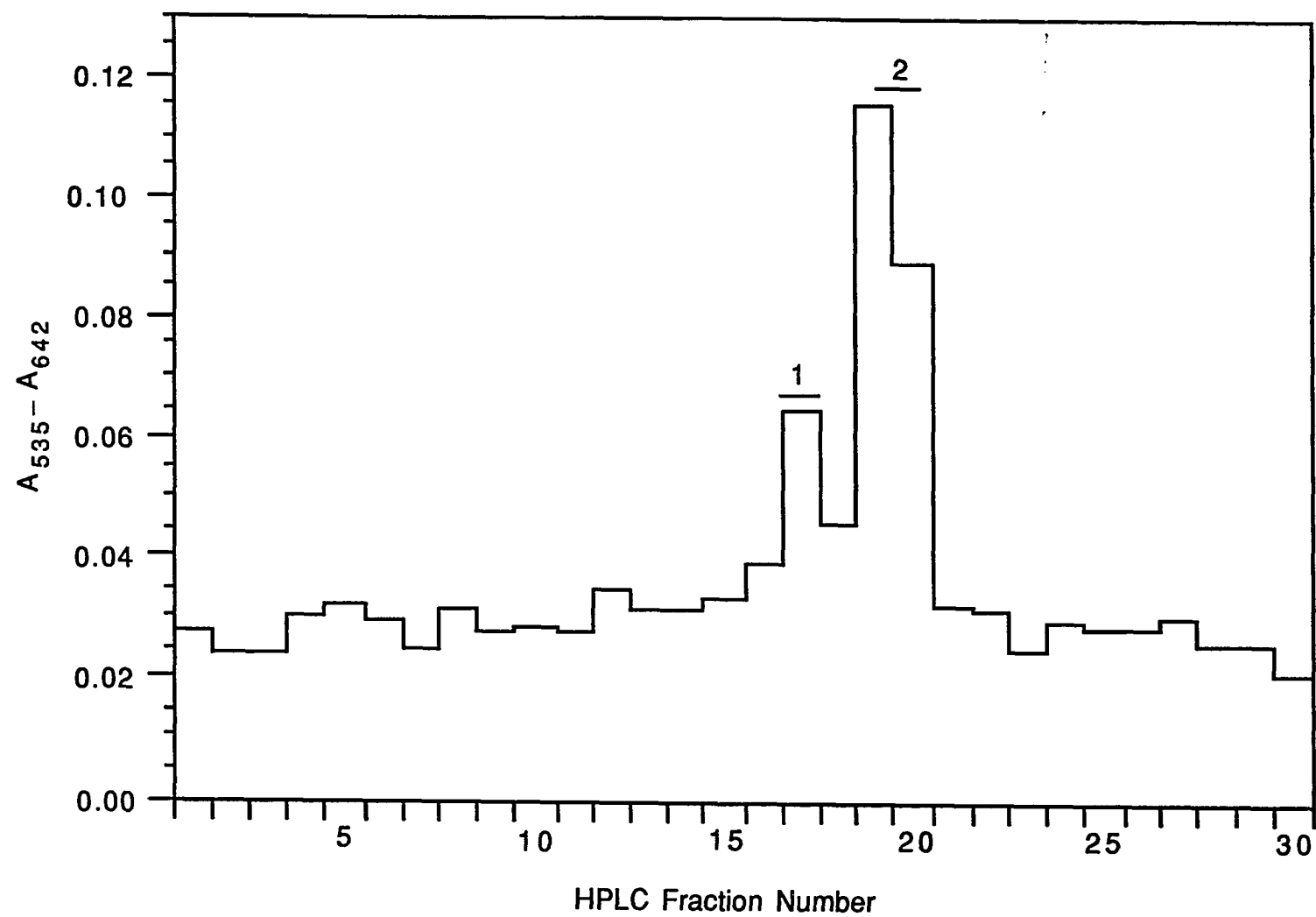


Figure 5. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 17-19 following HPLC purification. Known standards eluted in the following regions: 1, Z and 2, ZR

Figure 5. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 17-19 following HPLC purification. Known standards eluted in the following regions: 1, Z and 2, ZR

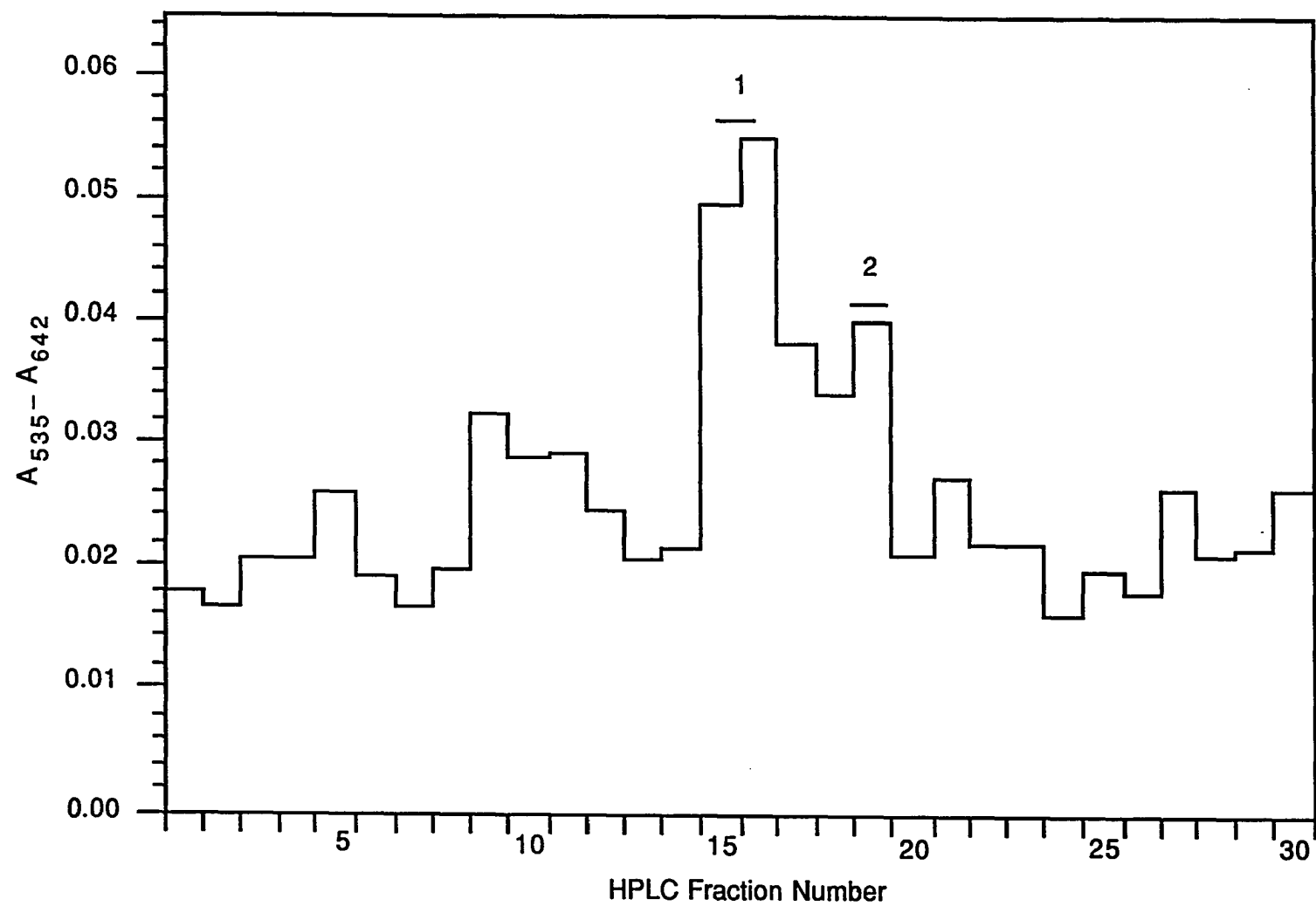


Figure 6. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 11-13 from the acidic fraction of a 650-g-FW extract following HPLC purification. The acidic fraction was treated with acid phosphatase before LH-20 chromatography but the residue of the LH-20 fractions was not treated with β -glucosidase before HPLC purification. Known standards eluted in the following regions: 1, Z9G; 2, ZOG; and 3, ZR/DHZROG

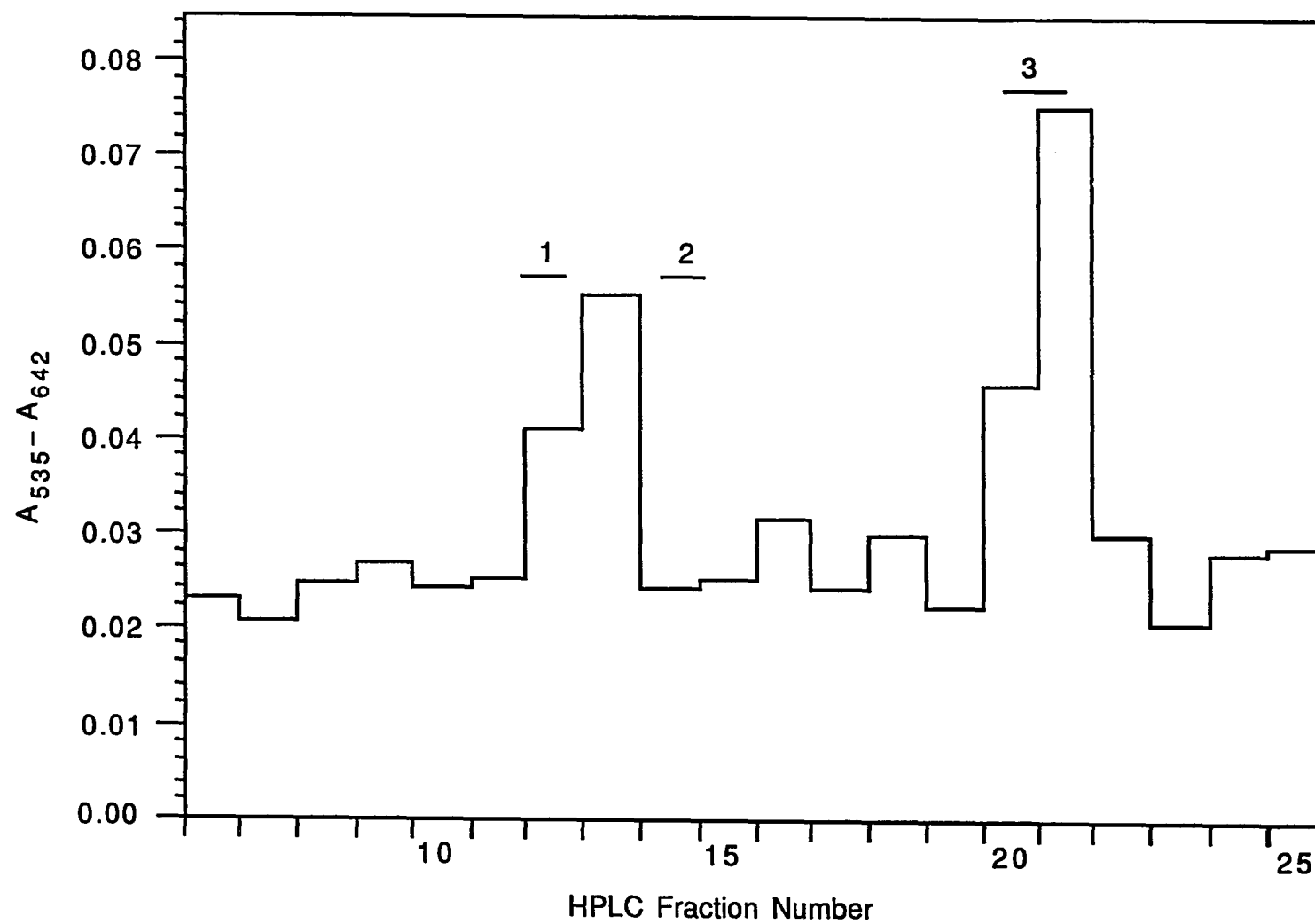
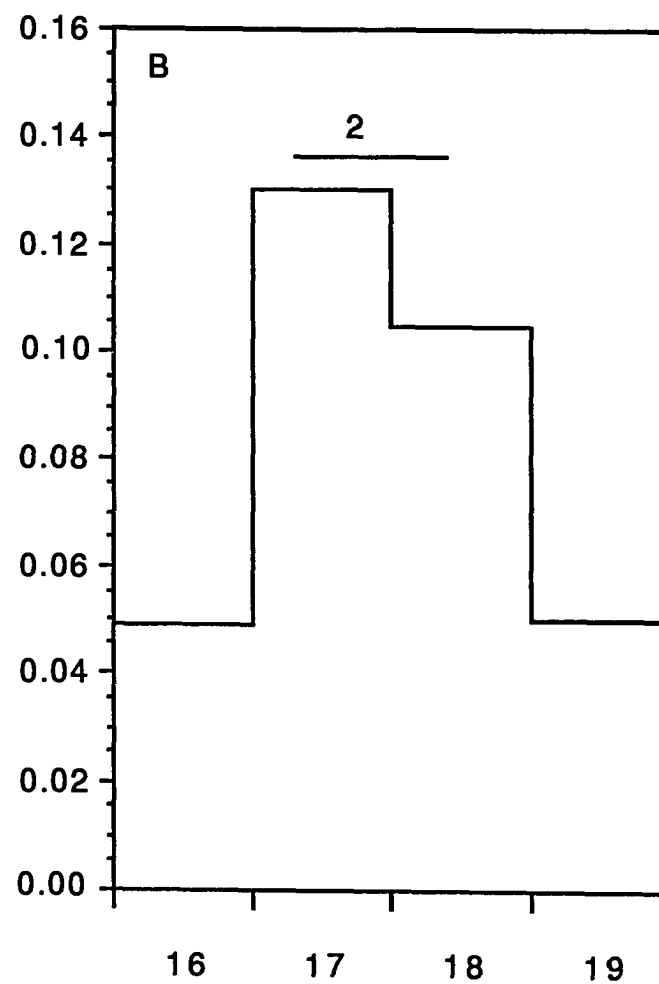
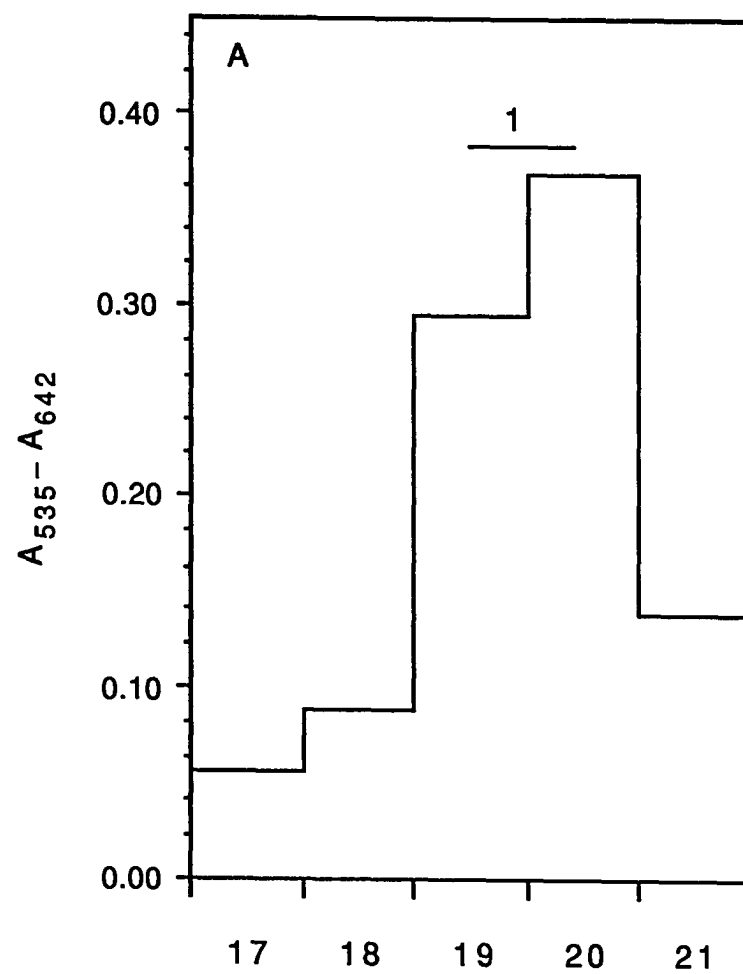


Figure 7. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 14-16 (A) and 22-24 (B) from the acidic fraction of a 650-g-FW extract following HPLC purification. The acidic fraction was treated with acid phosphatase before LH-20 chromatography. Known standards eluted in the following regions: 1, ZR and 2, iPA. The sample containing biological activity at the retention time of iPA on the LH-20 column was chromatographed on ODS-2 (B) using a linear gradient of 8-30% acetonitrile over 20 min.



HPLC Fraction Number

GC-MS Analysis

The HPLC trace from the run used to purify Me-ZR is shown in Figure 8. Only the HPLC fractions which co-chromatographed with the Me-ZR standard on ODS-2 contained sufficient CK to obtain a mass spectrum. The total ion chromatogram (TIC) from the GC-MS analysis of the HPLC fractions collected at the retention time of Me-ZR can be seen in Figure 9. The mass spectra obtained at the retention time of Me-ZR for both the plant sample and an authentic Me-ZR standard are shown in Figures 10 and 11, respectively. The characteristic ion fragments, and their relative abundances, of Me-ZR (M^+ 421(6), 390(71), 348(6), 216(100), 174(17), 148(6), 101(11), 71(10), 67(8), 45(45) and 41(17)) reported by Horgan and Scott (1987) are similar to those seen in both mass spectra, particularly at the higher masses. This confirms the presence of ZR in red pine shoots.

Figure 8. The UV absorbance of the plant sample, which had the retention time of ZR on ODS-2, was subsequently permethylated and re-chromatographed isocratically in 30% acetonitrile on ODS-2. The horizontal bar labelled "1" indicates the region within which a Me-ZR standard elutes under the same conditions. Full scale =0.05 AU

Figure 8. The UV absorbance of the plant sample, which had the retention time of ZR on ODS-2, was subsequently permethylated and re-chromatographed isocratically in 30% acetonitrile on ODS-2. The horizontal bar labelled "1" indicates the region within which a Me-ZR standard elutes under the same conditions. Full scale =0.05 AU

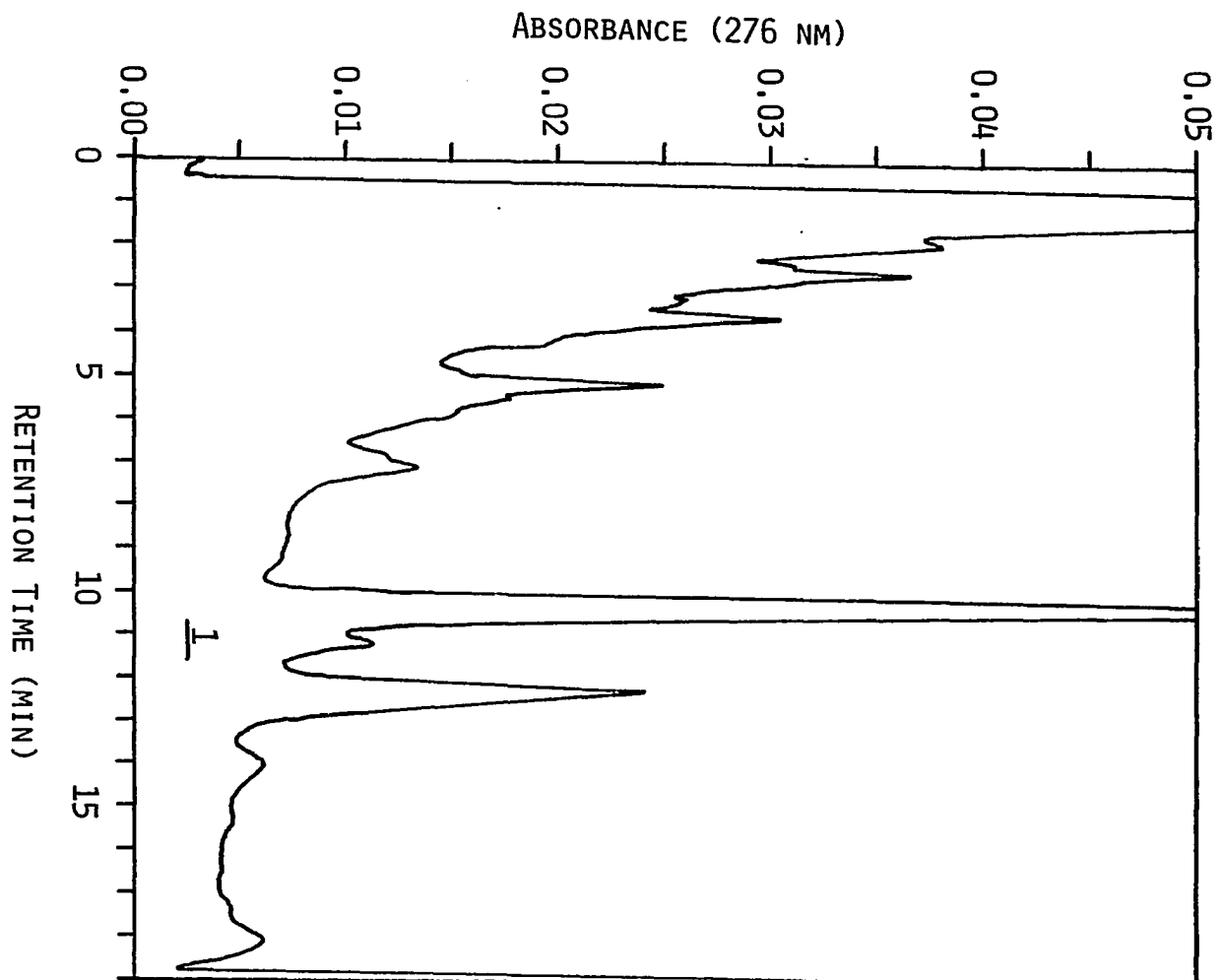
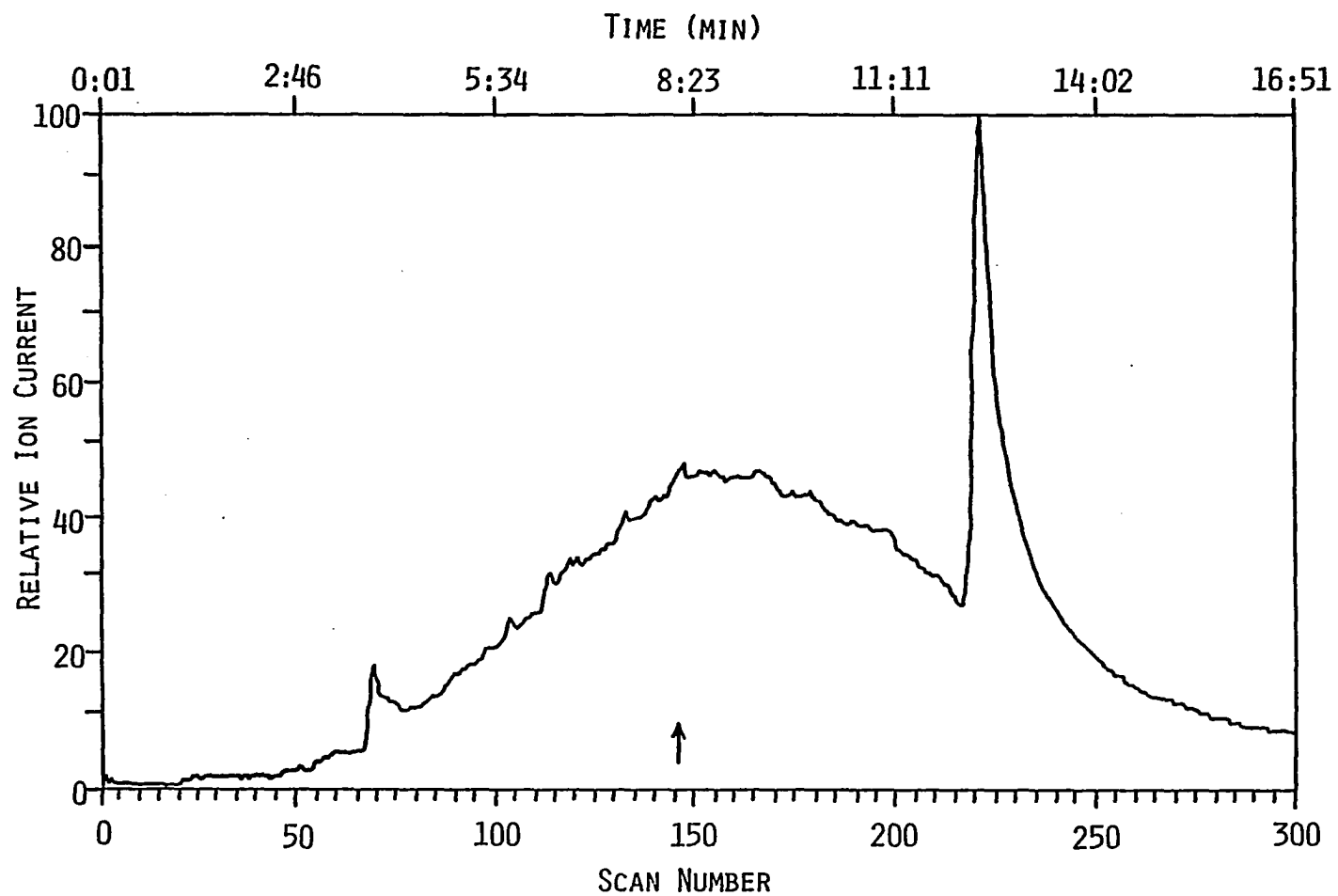


Figure 9. Total ion chromatogram from the GC-MS analysis of the permethylated plant sample which co-chromatographed with an authentic Me-ZR standard on ODS-2. The range of masses scanned was 27-490 amu. The arrow indicates the retention time of a Me-ZR standard. 100%=4.84 X 10⁷ counts



**Figure 10. Mass spectrum obtained from scan #146 of the TIC shown in
Figure 9**

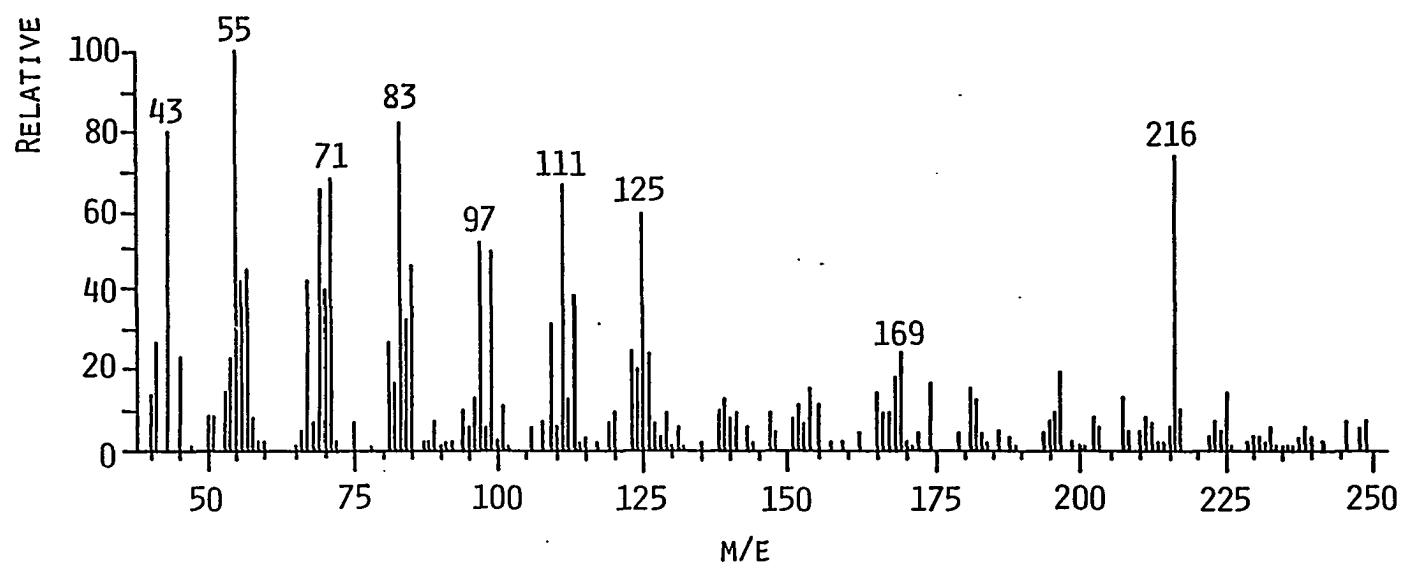
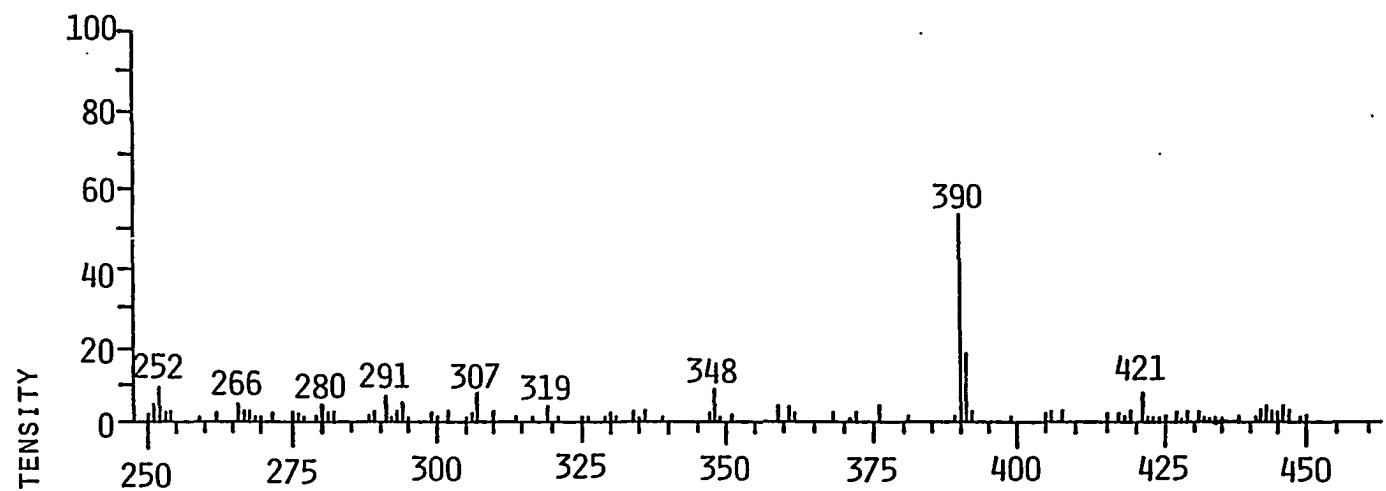
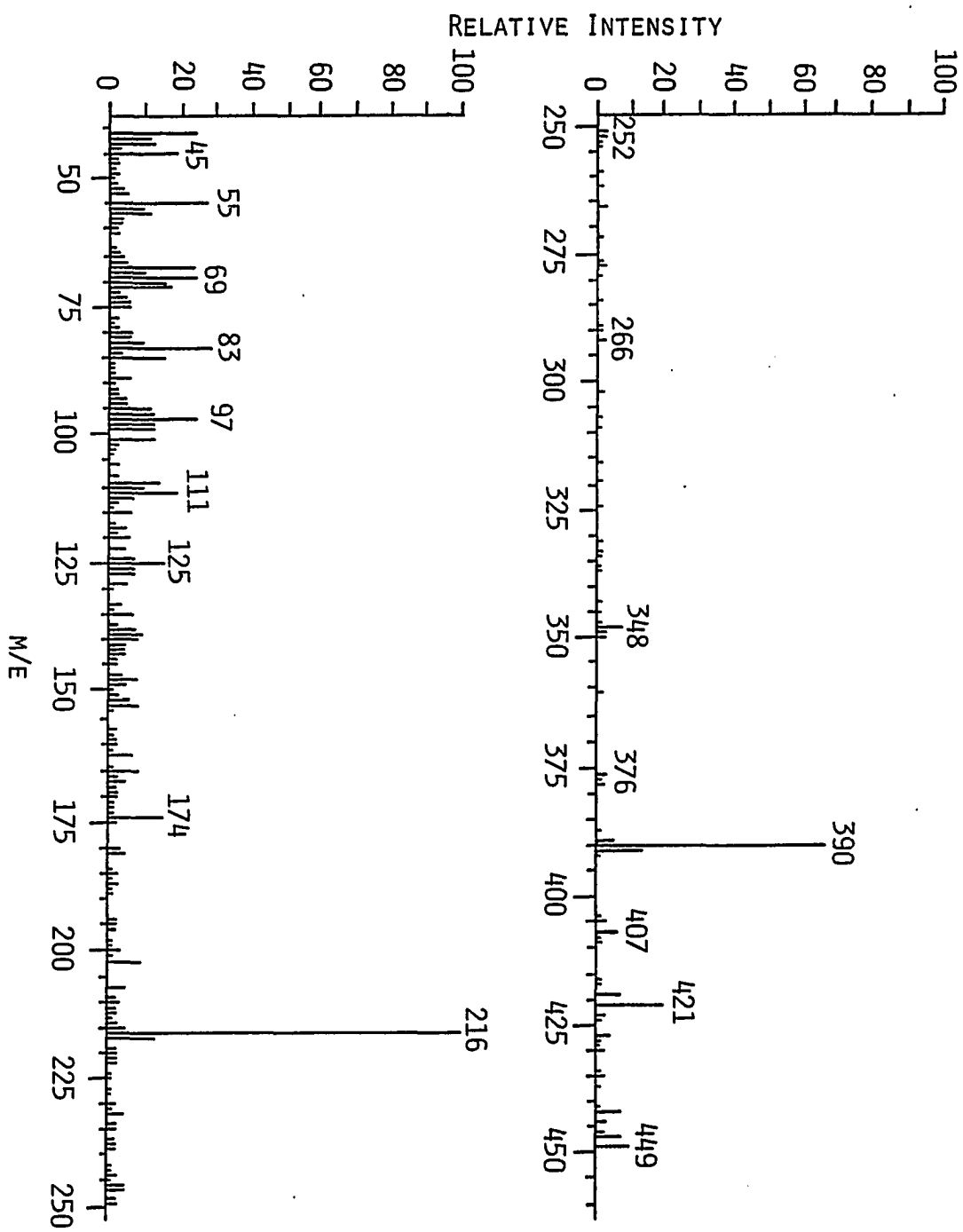


Figure 11. Mass spectrum of an authentic Me-ZR standard



DISCUSSION

The first peak of biological activity (fractions 11-13) from the LH-20 run used to purify the shoot basic fraction co-chromatographed with a DHZOG standard on an ODS-2 column (Fig. 2). When the residue of the equivalent LH-20 fractions from the 23-g-DW extract was treated with β -glucosidase before HPLC purification, two peaks of biological activity were detected. One corresponded to the retention time of DHZOG and the other to that of DHZ (Fig. 3). Although Z and DHZOG have nearly identical retention times on ODS-2, under the conditions used, free bases and glucosides resolve completely on LH-20. Because the free bases elute so much later than the glucosides (elution volumes of 350-400 cm³ and 550-625 cm³, respectively; Horgan and Scott 1987), the former should be absent from the glucoside-containing fractions (11-13). Hence, the first peak of biological activity seen in Fig. 3 cannot be due to endogenous Z.

Initially it was thought that the peak of biological activity detected at the retention time of DHZOG (and Z) could have been due to endogenous ZOG that had been converted to Z via glucosidase treatment. However, ZOG and DHZOG resolve completely on ODS-2 (Fig. 3), and only a single peak of biological activity was detected before β -glucosidase treatment. Therefore, the two peaks of biological activity seen in Fig. 3 must be due to incomplete cleavage of DHZOG.

When the residue of combined LH-20 fractions 14-16, from the run used to purify the basic fraction, was chromatographed on ODS-2, two peaks of biological activity were detected (Fig. 4). The major peak of activity had the same retention time as ZR, the minor peak of activity was at the retention time of DHZOG (and Z). With a flow rate of 30 cm³ h⁻¹, CK glucosides elute from LH-20 between 11.7 and 13.3 h. Because LH-20 fractions were collected at one-hour intervals and fractions 14-16 were pooled for analysis of the riboside, the minor peak could be

due to the presence of glucoside in the riboside sample. Alternatively, the minor peak of biological activity could be due to incomplete separation of the riboside and free base on LH-20. (Z and DHZOG have the same retention time on ODS-2.)

Similarly, two peaks of biological activity were detected in the HPLC run used to purify the residue of LH-20 fractions 17-19 (see Fig. 5). The first was present at the retention time of Z, the second at that of ZR. Once again, this is most likely due to the incomplete separation of the riboside and free base on LH-20.

Because the pH optimum for acid phosphatase is closer to the pH of the acidic fraction than that of alkaline phosphatase, the former was used for treatment of the ribotides in the acidic fraction. Preliminary work showed that both enzymes were severely inhibited by some unknown component of the extract. Initially this inhibition was attributed to polyphenolics. However, the problem persisted even after the acidic fraction was passed through a PVP column. Assuming that whatever was inhibiting enzymatic activity was binding non-specifically, it was hoped that by adding a gratuitous protein acceptor (i.e., BSA) to the buffer in which the acidic fraction residue was dissolved, a few hours before the enzyme, activity would be restored. This was successful to a limited extent, but the treatment had to be repeated a few times in order to get a majority of the radioactivity (originally added as [2-³H]-AMP) to elute in the basic fraction of the cellulose phosphate column used to purify the reaction mixture.

The acidic fraction so treated was subsequently chromatographed on LH-20. Bioassay of the HPLC fractions from the run used to purify the residue of LH-20 fractions 11-13 revealed two peaks of biological activity. The first eluted somewhere between the retention times of Z9G and ZOG. Only half of the residue

from these HPLC fractions was bioassayed, the remainder was treated with β -glucosidase and re-chromatographed on ODS-2. Unfortunately, there was insufficient CK in the resulting HPLC fractions to determine if a shift in biological activity had occurred. (β -glucosidase will only cleave O-glucosides.) Although its identity is still unknown, this provides strong evidence for the existence of a CK glucosyl phosphate (a "glucotide") in the shoots of red pine seedlings.

The second peak of biological activity present in Figure 6 has the retention time of ZR. Unfortunately, ZR and dihydrozeatin 9-riboside O-glucoside (DHZR-OG) are also inseparable on ODS-2 under the conditions used. Therefore, this second peak of biological activity could again be due to the incomplete separation of the glucoside and riboside (glucosyl phosphate and ribotide, respectively, before phosphatase treatment) on LH-20 or to yet another glucosyl phosphate derivative.

When the residue of the riboside fractions from the LH-20 run used to purify the shoot acidic fraction was run on ODS-2 and bioassayed, a large peak of biological activity occurred at the retention time of ZR (Fig. 7A). When the residue of the LH-20 fractions corresponding to the retention time of iPA, from the same run, was purified on ODS-2, bioassay detected a peak of biological activity at the retention time of iPA (Fig. 7B). Because this activity was detected after treatment with acid phosphatase, zeatin 9-riboside monophosphate (ZMP) and isopentenyladenosine monophosphate (iPMP) appear to be present in the shoots of red pine seedlings. Because plant cells are impermeable to CK ribotides, the ribotides are rapidly converted to ribosides (Laloue and Pethe 1982) and the ribosides are considered to be transport forms of CK (Van Staden and Davey 1979), the presence of iPMP in the shoots of red pine suggests that CK biosynthesis is occurring there. This finding reinforces a report by Chen and Petschow

(1978) in which evidence was presented for the existence of CK biosynthesis in rootless tobacco plants (derived from tissue culture). Subsequently, Chen et al. (1985) demonstrated CK biosynthesis in isolated cambial cells of carrot. To localize the origin of iPMP in red pine shoots, this qualitative assessment needs to be repeated on leaf and stem tissue separately.

Despite the extensive purification procedure used, there were high levels of background in all the plant samples analyzed by GC-MS. Apparently these contaminants do not absorb UV light because the traces from the HPLC runs used to purify the plant samples (for example see Fig. 8) provide no indication of the level of contamination seen in the TIC (for example see Fig. 9). In future work with pine, a method for removing these interfering compounds must be found. One possibility involves using normal-phase HPLC purification of the permethyl derivatives (Horgan and Scott 1987). However, the addition of yet another step in the purification procedure will lead to further losses of sample. Purifying cytokinins from plant extracts using immunoaffinity chromatography (Hansen et al. 1989; MacDonald and Morris 1985) provides a more desirable alternative because it circumvents the need for some of the other purification steps described herein. This technique should be explored fully.

Finally, because so many workers are using enzyme-linked immunosorbent assays (ELISAs) to quantify CKs, a word of caution is in order. The antibodies used in most ELISA systems cross-react to various extents with a range of CKs. Due to the number of CKs present in pine, and other species, it is essential to separate all the CKs present in a given tissue before quantification by ELISA, if meaningful results are to be obtained.

REFERENCES

- Alvim, R., E. W. Hewett, P. F. Saunders. 1976. Seasonal variation in the hormone content of willow. I. Changes in abscisic acid content and cytokinin activity in the xylem sap. *Plant Physiology* 57: 474-476.
- Andersen, R. A. and J. A. Sowers. 1968. Optimum conditions for bonding of plant phenols to insoluble polyvinylpyrrolidone. *Phytochemistry* 7: 293-301.
- Biddington, N. L. and T. H. Thomas. 1973. A modified *Amaranthus* betacyanin bioassay for the rapid determination of cytokinins in plant extracts. *Planta* 111: 183-186.
- Bielecki, R. L. 1964. The problem of halting enzyme action when extracting plant tissues. *Analytical Biochemistry* 9: 431-442.
- Bollmark, M. and L. Eliasson. 1986. Effects of exogenous cytokinins on root formation in pea cuttings. *Physiologia Plantarum* 68: 662-666.
- Burrows, W. J. and D. J. Carr. 1969. Effects of flooding the root system of sunflower plants on the cytokinin content in the xylem sap. *Physiologia Plantarum* 22: 1105-1112.
- Chen, C.-m., J. R. Ertl, S. M. Leisner, C.-C Chang. 1985. Localization of cytokinin biosynthetic sites in pea plants and carrot roots. *Plant Physiology* 78: 510-513.
- Chen, C.-m. and B. Petschow. 1978. Cytokinin biosynthesis in cultured rootless tobacco plants. *Plant Physiology* 62: 861-865.
- Drew, A. P. 1982. Shoot-root plasticity and episodic growth in red pine seedlings. *Annals of Botany* 49: 347-357.
- Engelbrecht, L. 1972. Cytokinins in leaf-cuttings of *Phaseolus vulgaris* L. during their development. *Biochemie und Physiologie der Pflanzen* 163: 335-343.
- Eriksen, E. N. 1974. Root formation in pea cuttings. III. The influence of cytokinins at different developmental stages. *Physiologia Plantarum* 30: 163-167.
- Feldman, L. J. 1975. Cytokinins and quiescent center activity in roots of *Zea*. Pages 55-72 in J. G. Torrey and D. T. Clarkson, eds. *The development and function of roots*. Academic Press, New York.

- Hansen, C. E., C. Kopperud, and O. M. Heide. 1989. Immunoaffinity chromatography for purification of cytokinins in plant extracts. *Norwegian Journal of Agricultural Science* 3: 73-77.
- Horgan, R. and M. R. Kramers. 1979. High-performance liquid chromatography of cytokinins. *Journal of Chromatography* 173: 263-270.
- Horgan, R. and I. M. Scott. 1987. Cytokinins. Pages 303-365 in L. Rivier and A. Crozier, eds. *Principles and practices of plant hormone analysis*. Academic Press, New York.
- Laloue, M. and C. Pethe. 1982. Dynamics of cytokinin metabolism in tobacco cells. Pages 185-195 in P. F. Wareing, ed. *Plant growth substances 1982*. Academic Press, London, U. K.
- MacDonald, E. M. S. and R. O. Morris. 1985. Isolation of cytokinins by immunoaffinity chromatography and analysis by high-performance liquid chromatography radioimmunoassay. *Methods in Enzymology* 110: 347-358.
- Meilan, R., R. C. Schultz, and C. E. LaMotte. 1987. Manipulating foliar development in red pine through exogenous applications of cytokinin. *Plant Physiology* 83: 97, Supplement.
- Mozes, R. and A. Altman. 1977. Characteristics of root-to-shoot transport of cytokinin 6-benzylaminopurine in intact seedlings of *Citrus aurantium*. *Physiologia Plantarum* 39: 225-232.
- Palmer, M. V., R. Horgan, and P. F. Wareing. 1981a. Cytokinin metabolism in *Phaseolus vulgaris* L. Identification of endogenous cytokinins and metabolism of [8-¹⁴C]-dihydrozeatin in stems of decapitated plants. *Planta* 153: 297-302.
- Palmer, M. V., R. Horgan, and P. F. Wareing. 1981b. Cytokinin metabolism in *Phaseolus vulgaris* L. I: Variations in cytokinin levels in leaves of decapitated plants in relation to lateral bud outgrowth. *Journal of Experimental Botany* 32: 1231-1241.
- Purse, J. G., R. Horgan, J. M. Horgan, and P. F. Wareing. 1976. Cytokinins of sycamore spring sap. *Planta* 132: 1-8.
- Radin, J. W. and R. S. Loomis. 1971. Changes in the cytokinins of radish roots during maturation. *Physiologia Plantarum* 25: 240-244.
- Schultz, R. C. and J. R. Thompson. 1990. Nursery practices that improve hardwood seedling root morphology. *Tree Planters' Notes* (in press).

- Scott, I. M. and R. Horgan. 1984. Root cytokinins of *Phaseolus vulgaris* L. Plant Science Letters 34: 81-87.
- Scott, I. M., R. Horgan, and B. A. McGaw. 1980. Zeatin-9-glucoside, a major endogenous cytokinin on *Vinca rosea* L. crown gall tissue. Planta 149: 472-475.
- Scott, T. K. 1972. Auxins and roots. Annual Review of Plant Physiology 23: 235-258.
- Short, K. C. and J. G. Torrey. 1972. Cytokinins in seedling roots of pea. Plant Physiology 49: 155-160.
- Skene, K. G. M. 1975. Cytokinin production by roots as a factor in the control of plant growth. Pages 365-396 in J. G. Torrey and D. T. Clarkson, eds. The development and function of roots. Academic Press, New York.
- Stenlid, G. 1982. Cytokinins as inhibitors of root growth. Physiologia Plantarum 56: 500-506.
- Van Staden, J. and J. E. Davey. 1979. The synthesis, transport and metabolism of endogenous cytokinins. Plant, Cell and Environment 2: 93-106.

**SECTION IV. IDENTIFICATION OF THE CYTOKININS IN RED PINE
SEEDLINGS. II. THE ROOTS**

SUMMARY

Zeatin 9-riboside has been identified in roots of *Pinus resinosa* by GC-MS analysis of its permethyl derivative and by its retention times on Sephadex LH-20 and Spherisorb ODS-2. Based on their retention times and susceptibility to enzymatic degradation, several other cytokinins have been tentatively identified. These include zeatin O-glucoside and zeatin in the basic fraction and zeatin 9-riboside monophosphate, isopentenyladenosine monophosphate (iPMP) and a glucosyl phosphate derivative in the acidic fraction. The latter compound appears to be a phosphorylated derivative of zeatin O-glucoside. The presence of iPMP supports the commonly held notion that cytokinin biosynthesis takes place in roots. The amount of iPMP detected was roughly equivalent to what was detected in red pine shoots. Either cytokinin biosynthesis is coordinated between the roots and shoots or the rate of cytokinin export from the roots is matched by shoot metabolism to maintain a steady-state level.

INTRODUCTION

In the first article of this two-part series, the cytokinins (CKs) found in the shoots of red pine (*Pinus resinosa* Ait.) seedlings were described. This paper reports on the CKs identified in the corresponding root material.

MATERIALS AND METHODS

The materials and methods used for characterizing the CKs in the roots of red pine were identical to those used for the shoots. The only difference was in the amount of tissue extracted. Fifty-six grams dry weight (g DW; equivalent to 135 grams fresh weight) of root material was extracted to determine the retention times of the basic fraction CKs on LH-20 and ODS-2 (as opposed to 115 grams fresh weight (g FW) for the shoots). Acidic fraction CKs were characterized using the equivalent of 410 g FW of root material. In an attempt to obtain mass spectra of the basic fraction CKs, the purification process was repeated using 315 g DW (equivalent to 755 g FW) of root material.

RESULTS

Basic Fraction

As with the shoots, three zones of biological activity were detected when half of the LH-20 fractions from the run used to purify the 135-g-FW extract were bioassayed. (Fig. 1). When the other half of LH-20 fractions 11-13 were combined and purified on ODS-2, a bioassay of the HPLC fractions revealed two major peaks of biological activity (Fig. 2). One of these peaks of activity eluted earlier than the most polar standard used, zeatin 7-glucoside (Z7G); the other co-chromatographed with Z. When the residue from the combined LH-20 fractions 11-13 of the 755-g-FW extract was treated with β -glucosidase before chromatographic purification on ODS-2, two major peaks of biological activity were seen (Fig. 3). The first of these peaks had the retention time of Z, the other of DHZ. There was also a minor peak of activity at the retention time of ZR.

Bioassay of the HPLC fractions from the runs used to purify the residue from the remainder of LH-20 fractions 14-16 and 17-19 each revealed two prominent peaks of biological activity. Once again, the first peak of activity from LH-20 fractions 14-16 eluted from ODS-2 before the most mobile standard used (Z7G); the second peak had the same retention time as ZR (Fig. 4). The two peaks of biological activity detected in LH-20 fractions 17-19 eluted from ODS-2 at the same retention times as Z and ZR (Fig. 5).

Acidic Fraction

A single peak of biological activity was detected in the HPLC fractions from the run in which the residue of LH-20 fractions 11-13 was purified (Fig. 6). This occurred at the retention time of zeatin O-glucoside (ZOG). After treating the

Figure 1. *Amaranthus* betacyanin bioassay profile of the basic fraction from the 135-g-FW extract following LH-20 chromatography. A 78 X 2.5 cm column was eluted with 35% EtOH at a flow rate of 30 cm³/hr. Fractions were collected at one-hour intervals. Authentic standards eluted in the following regions: 1, CK glucosides; 2, CK ribosides; and 3, CK free bases. Asterisks indicate fractions containing [³H]-DHZ; the number of asterisks is proportional to the activity present. Fractions 11-13 were combined for further purification, as were 14-16 and 17-19

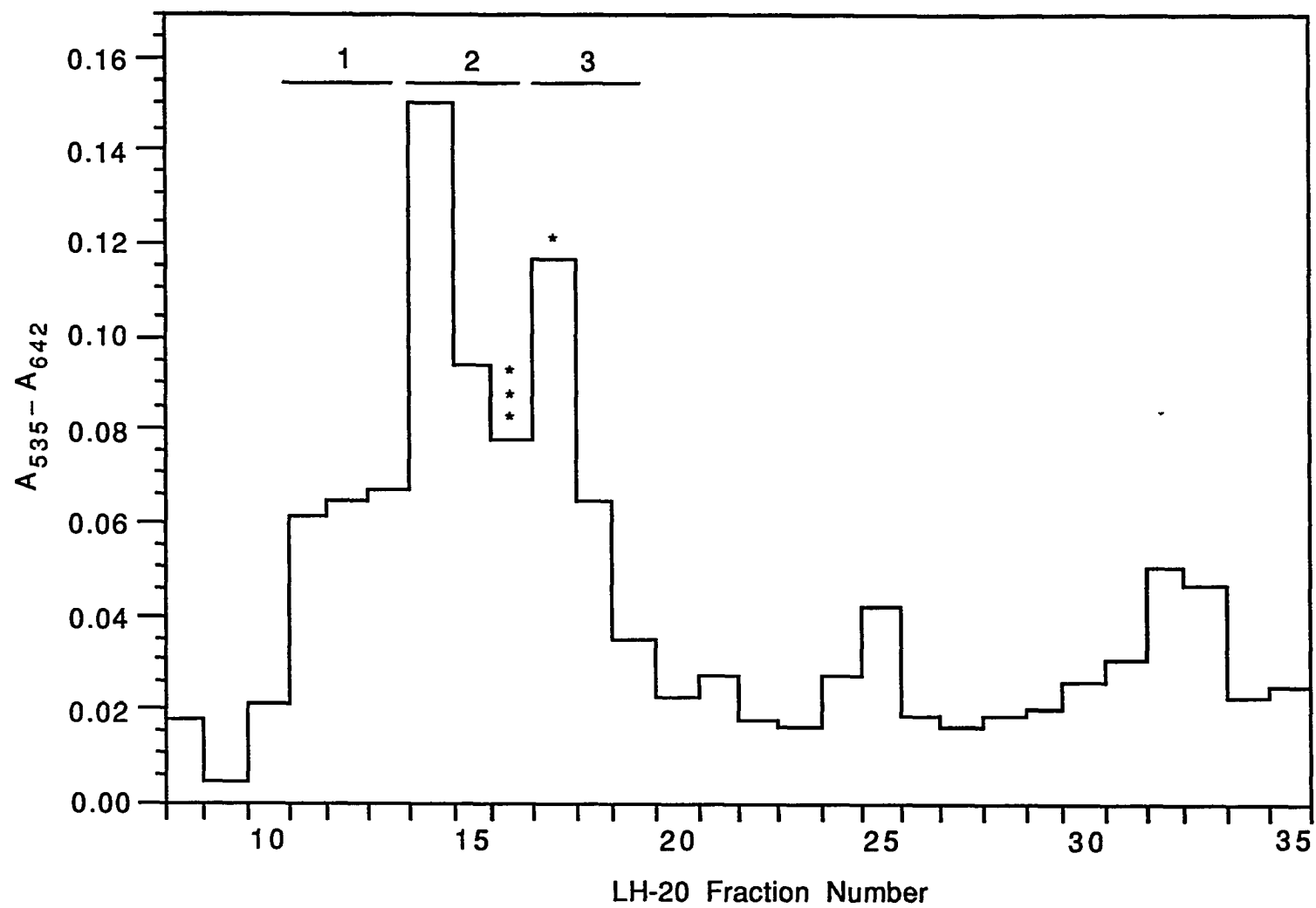


Figure 2. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 11-13 following HPLC purification. Unless otherwise indicated a 150 mm X 4.5 mm column of ODS-2 was eluted with H₂O containing a linearly increasing concentration of acetonitrile (5-20% over 40 min.) at a flow rate of 2.0 cm³/min. Fractions were collected at one-minute intervals. Authentic standards eluted in the following regions: 1, Z7G; 2, ZOG; and 3, Z/DHZOG

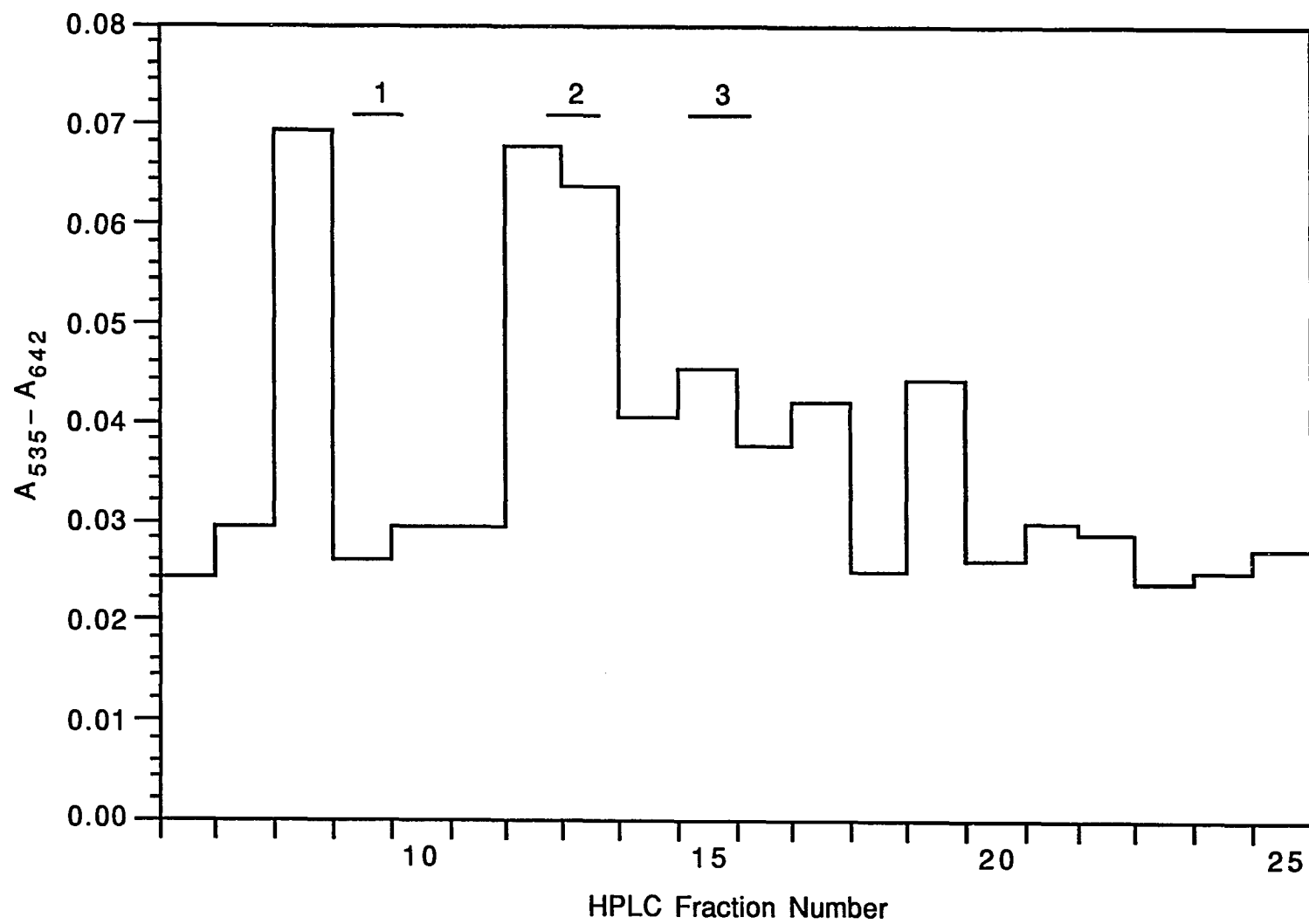


Figure 3. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 11-13 after treatment with β -glucosidase and HPLC purification. Known standards eluted in the following regions: 1, Z/DHZOG; 2, DHZ; 3, ZR and 4, DHZR

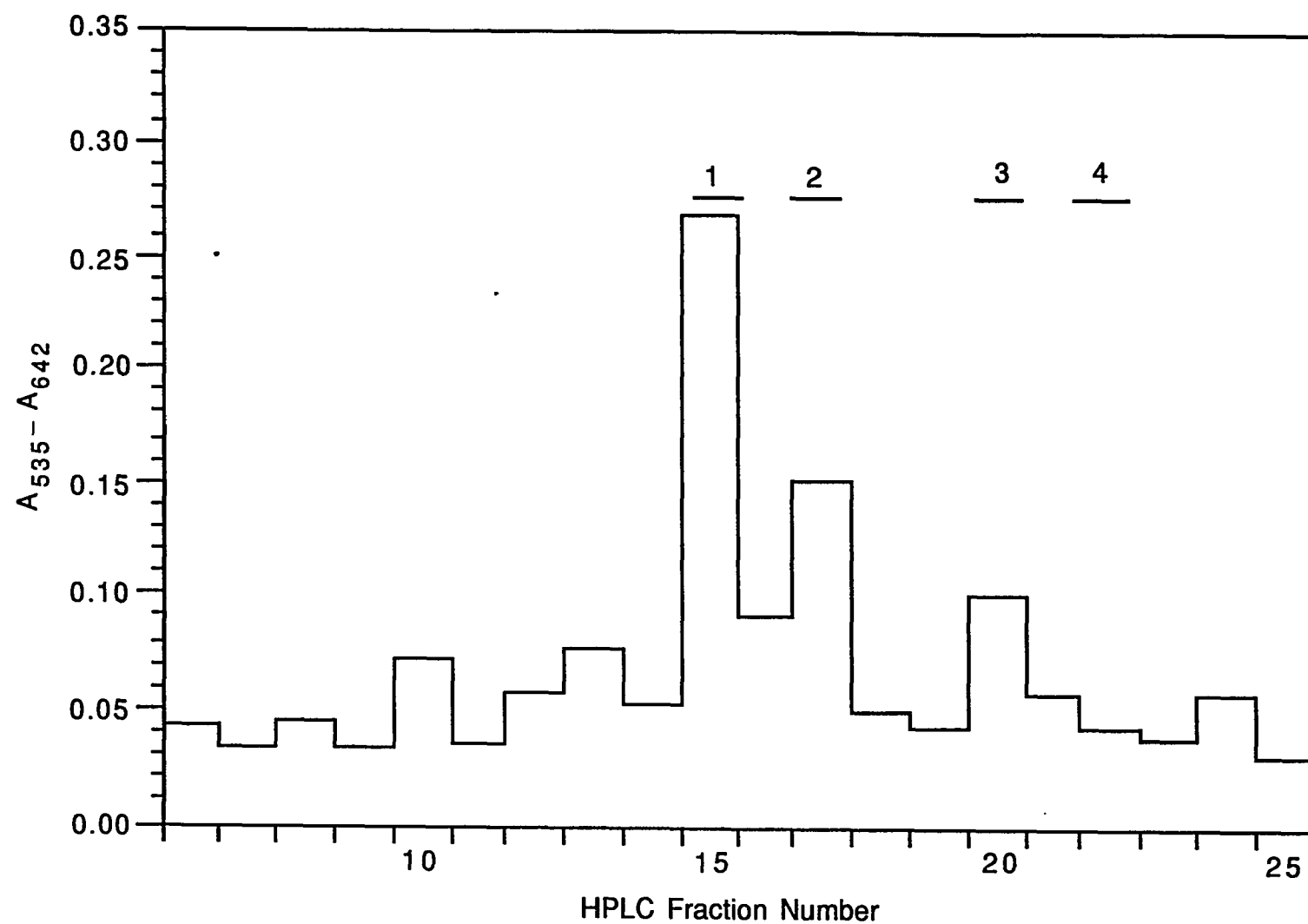


Figure 4. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 14-16 following HPLC purification. Known standards eluted in the following regions: 1, Z7G and 2, ZR

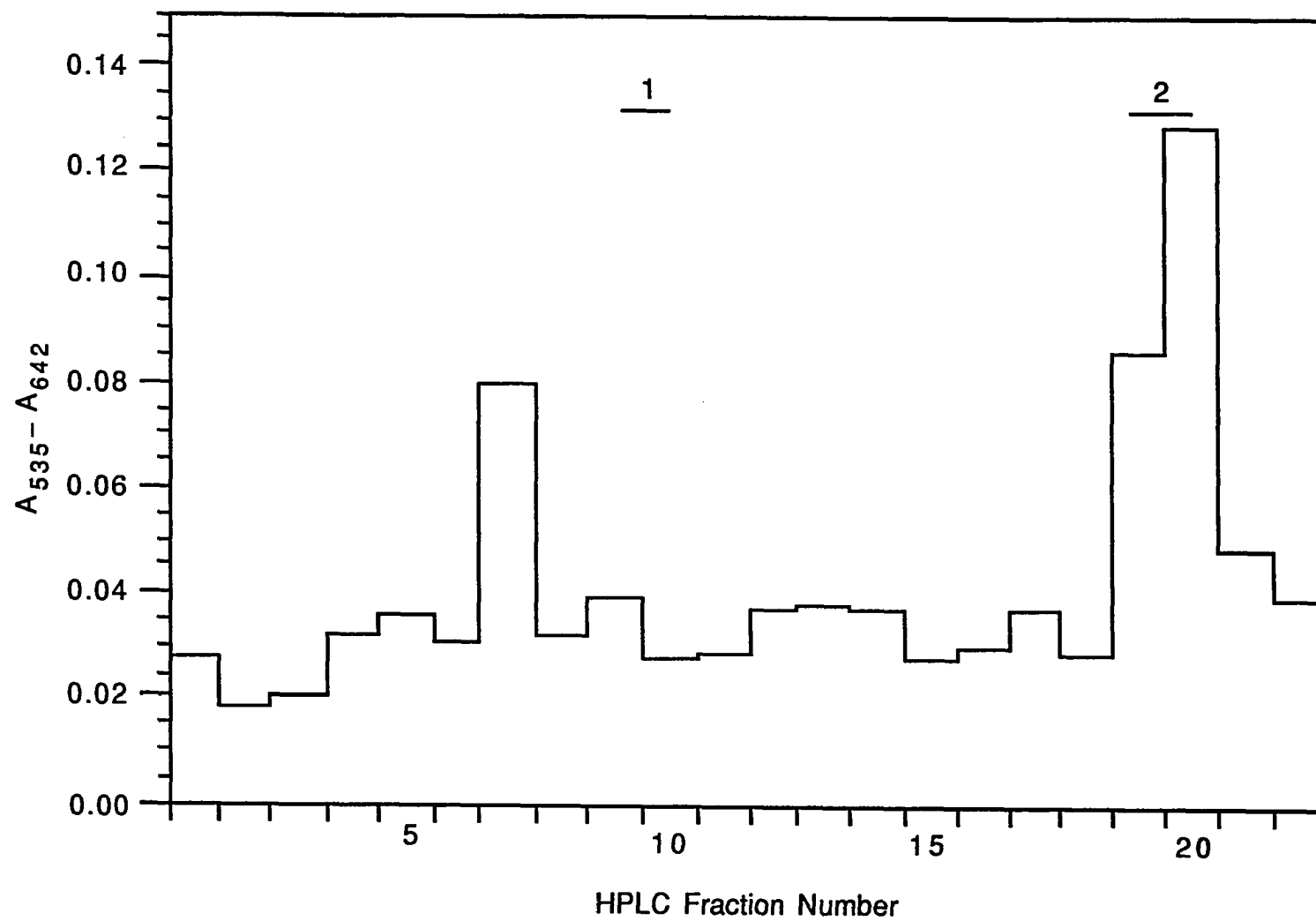


Figure 5. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 17-19 following HPLC purification. Known standards eluted in the following regions: 1, Z and 2, ZR

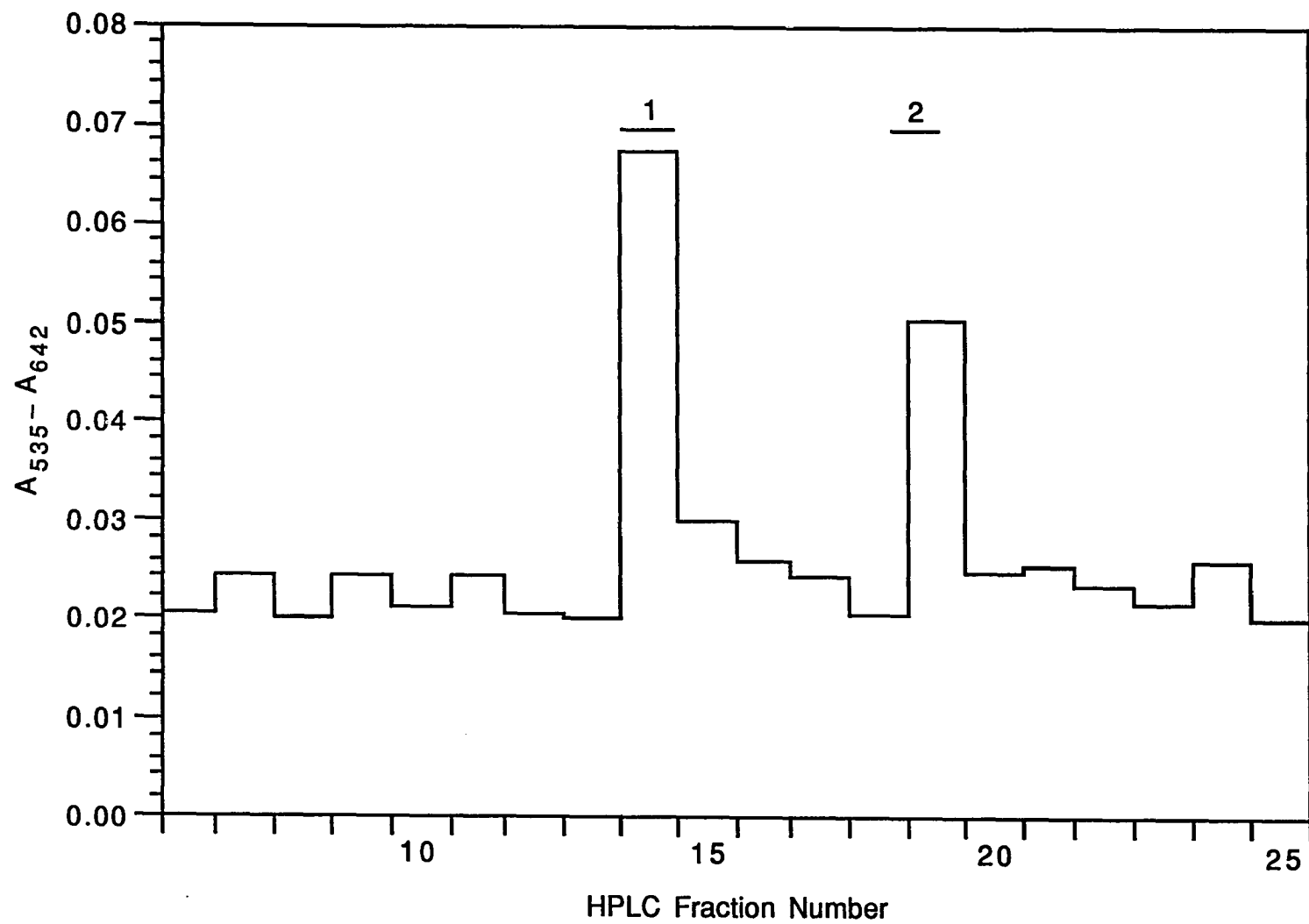
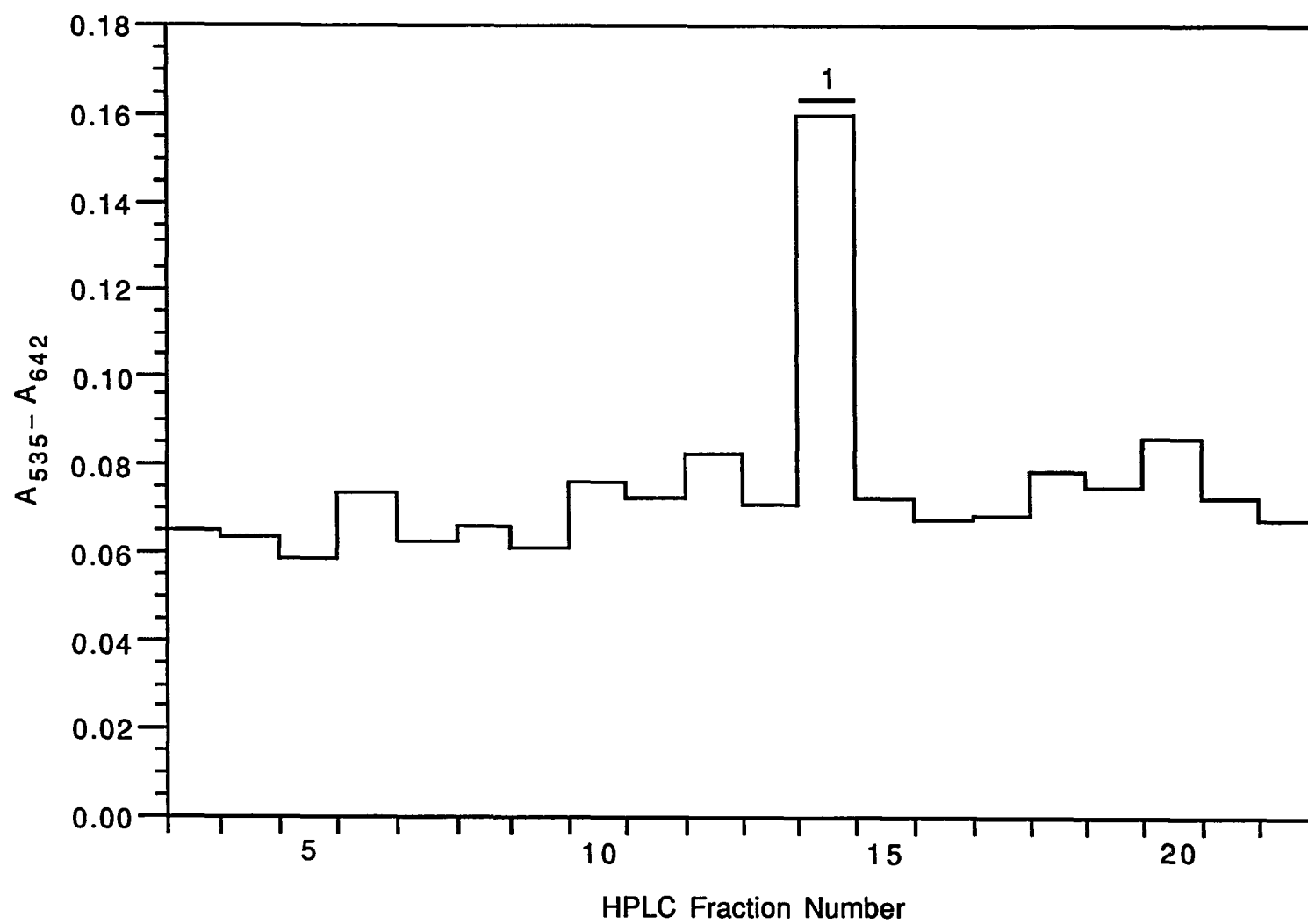


Figure 6. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 11-13 from the acidic fraction of a 410-g-FW extract following HPLC purification. The acidic fraction was treated with acid phosphatase before LH-20 chromatography but was not treated with β -glucosidase before HPLC purification. An authentic ZOG standard eluted in the region labelled "1"

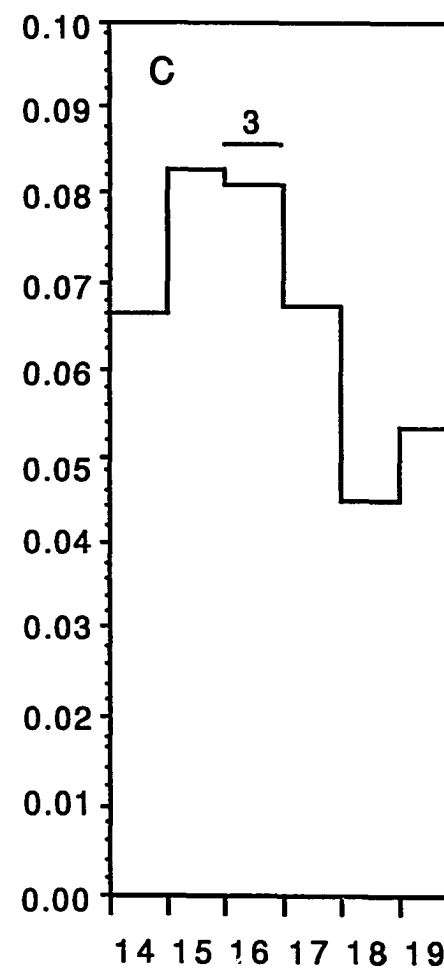
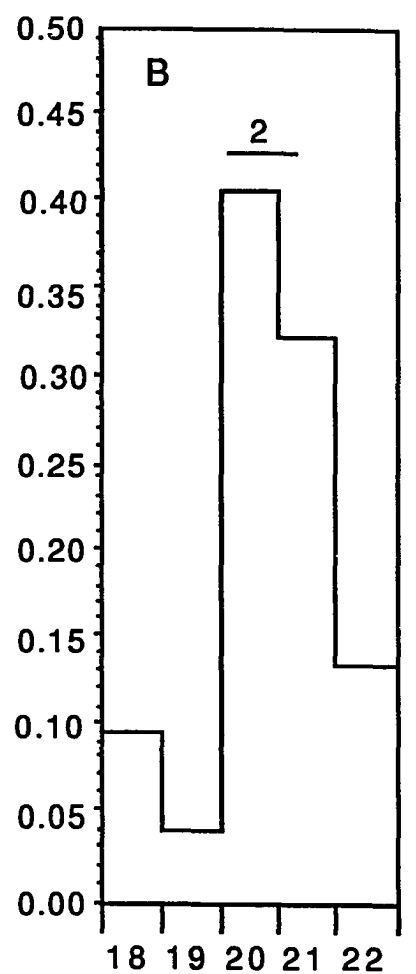
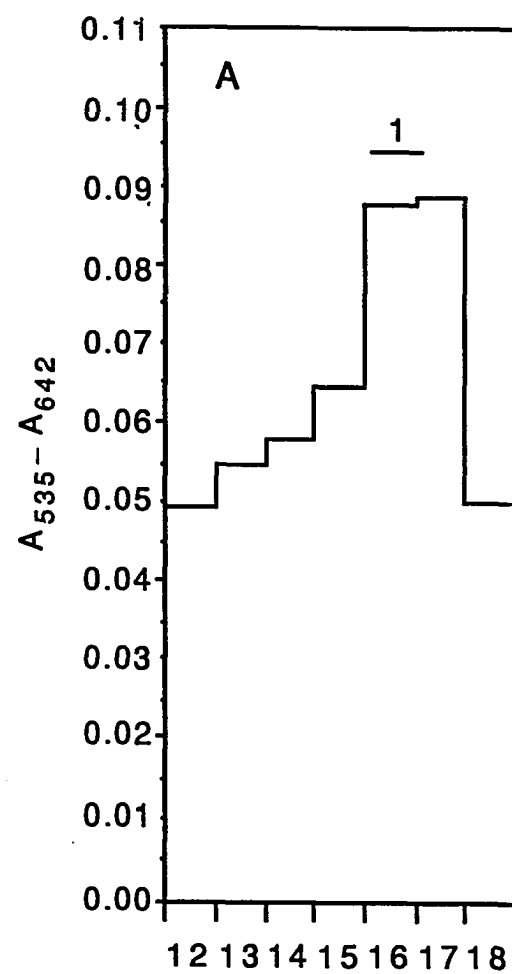


other half of this sample with β -glucosidase, the activity shifted to the retention time of Z on ODS-2 (Fig. 7A). Analytical HPLC and subsequent bioassay of LH-20 fractions 14-16 and 22-24 indicated that compounds co-chromatographing with ZR and iPA were responsible for the activity seen (Figs. 7B and 7C, respectively).

GC-MS Analysis

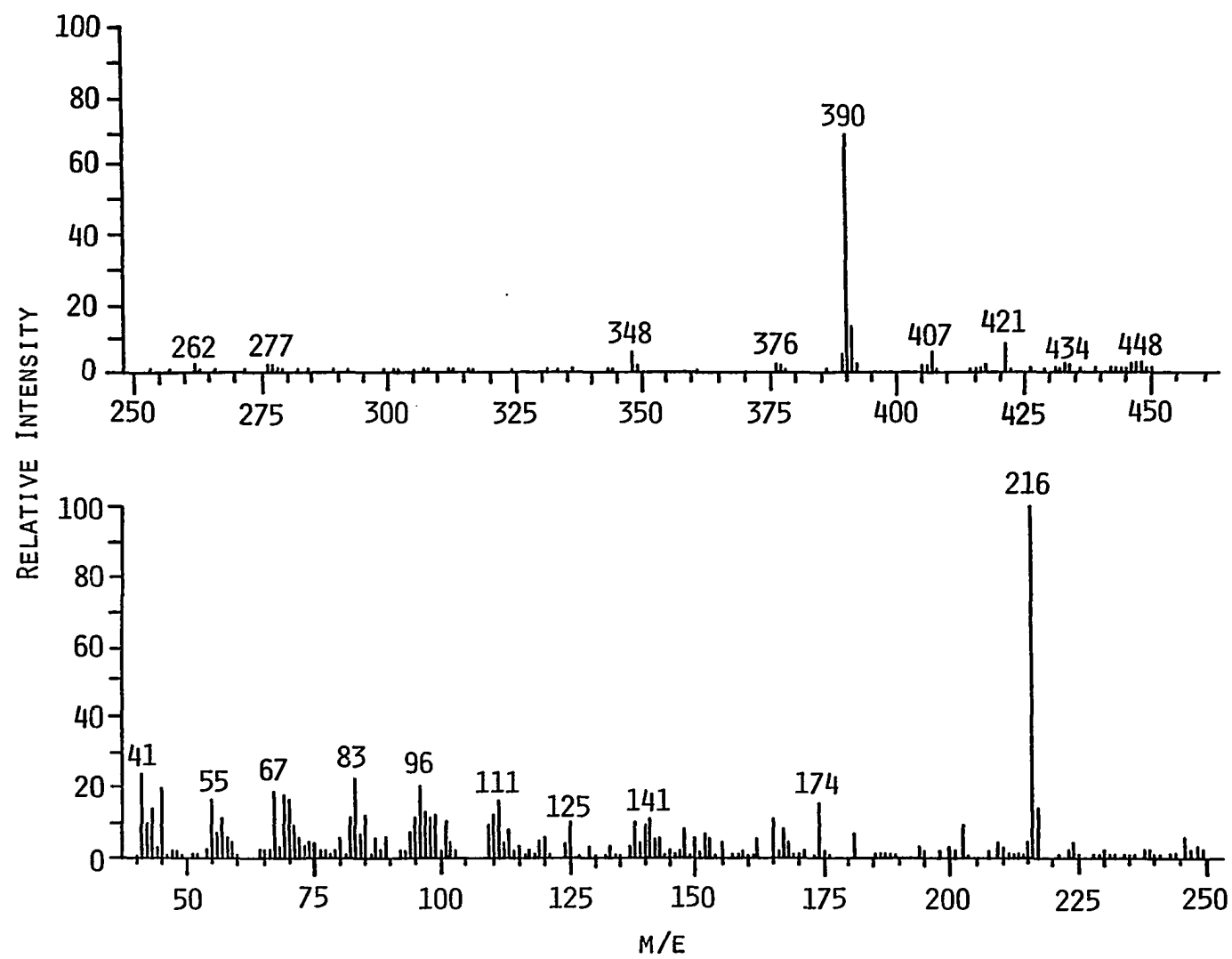
As with the shoot material, all root samples analyzed by GC-MS had high background levels. Only the sample from the basic fraction which co-chromatographed with a Me-ZR standard on ODS-2 and on the GC contained sufficient CK to obtain a mass spectrum (Fig. 8). The characteristic ion fragments, and their relative abundances, of Me-ZR (Fig. 11, Section III) are similar to those seen in Figure 8, particularly at the higher masses. This confirms the presence of ZR in red pine roots.

Figure 7. *Amaranthus* betacyanin bioassay profile of combined LH-20 fractions 11-13 (A), 14-16 (B) and 22-24 (C) from the acidic fraction of a 410-g-FW extract following HPLC purification. The acidic fraction was treated with acid phosphatase before LH-20 chromatography. The residue of fractions 11-13 was treated with β -glucosidase before HPLC purification. Known standards eluted in the following regions: 1, Z; 2, ZR; and 3, iPA. The sample containing biological activity at the retention time of iPA on the LH-20 column was chromatographed on ODS-2 (C) using a linear gradient of 8-30% acetonitrile over 20 min.



HPLC Fraction Number

Figure 8. Mass spectrum obtained from a basic fraction sample co-chromatographing with an authentic ZR standard on LH-20 and ODS-2 and with a Me-ZR standard during GC-MS analysis



DISCUSSION

It is apparent that the combined LH-20 glucoside fractions (11-13) contain at least two compounds with biological activity (Fig. 2). The second peak contains more biological activity than the first one. When the equivalent LH-20 fractions were treated with β -glucosidase before HPLC purification, the retention time of both peaks of biological activity shifted on ODS-2 (Fig. 3). Because the major peak of activity before enzymatic treatment co-chromatographed with ZOG, only O-glucosides are susceptible to β -glucosidase, and the major peak of activity detected after enzymatic treatment had the retention time of Z, it would appear that the endogenous compound responsible for this activity is, in fact, ZOG.

It has already been shown (Section III), that some CK-glucosides and CK-ribosides are not completely resolved on LH-20. Here again, there are two peaks of biological activity in the LH-20 fractions collected in the region corresponding to the retention time of CK-ribosides (fractions 14-16) when chromatographed on ODS-2 (Fig. 4). The second peak of activity had the retention time of ZR but the first peak eluted in the same region as the more polar glucoside present in LH-20 fractions 11-13 (peak 1, Fig. 2). This same peak of activity has also been seen in other red pine root extracts (data not shown); therefore, it is not an aberration. There is a biologically active compound that elutes from ODS-2 at a very low concentration of organic solvent in the mobile phase.

Due to its susceptibility to β -glucosidase and its behavior on LH-20, the compound in question appears to be an O-glucoside of some sort. It cannot be dihydrozeatin O-glucoside (DHZOG) because it elutes from ODS-2 much earlier than DHZOG before treatment with β -glucosidase (see Fig. 2). Based on its affinity for ODS-2, it possesses more polar character than either ZOG or Z7G. Taylor et al.

(1984) have described a novel ribosyl zeatin glycoside from *Pinus radiata* which has a glucosyl moiety attached to the ribosyl moiety of ZR. If the compound in question was of this nature, a peak of biological activity would have been expected at the retention time of ZR or dihydrozeatin riboside (DHZR) following treatment with β -glucosidase, not at the retention time of DHZ (see Fig. 3). Needless to say, much more work needs to be done to fully characterize this compound.

There also appears to be incomplete separation of the free bases and ribosides on LH-20, as seen with the glucosides and ribosides. When the residue of the LH-20 fractions corresponding to the retention time of free bases (17-19) was chromatographed on ODS-2, peaks of activity were observed at the retention times of Z and ZR (see Fig. 5). However, two independent observations of biological activity with the retention time of ZR help confirm its existence, especially when they were obtained from adjacent LH-20 fractions.

Although a putative CK glucoside phosphate (a "glucotide") was isolated from shoot material, nothing more could be said about its identity. As was done with the shoots, acidic fractions from several extracts were combined and repeatedly treated with acid phosphatase before further purification. The residue of the glucoside LH-20 fractions (11-13) contained one peak of biological activity, at the retention time of ZOG, following reversed-phase HPLC (see Fig. 6). After treating the other half of this same fraction with β -glucosidase and running it on ODS-2, the biological activity shifted to the retention time of Z (see Fig. 7). Thus the CK glucoside phosphate appears to be an O-glucotide of zeatin.

The biological activity, derived from the root acidic fraction, which co-chromatographed with an authentic iPA standard on both LH-20 and ODS-2, following treatment with acid phosphatase, implies that iPMP is present in red pine roots as well as the shoots. Although there was less biological activity detected at the

retention time of iPA in the root acidic fraction, less root tissue was extracted (410 vs 650 g FW). When the biological activity found in the roots and shoots are expressed on a per g FW basis, there appears to be nearly equivalent amounts of iPMP in roots and shoots. These results must be interpreted with care, as they were obtained from tissue harvested at a single time. However, it is possible that either CK biosynthesis is coordinated between the roots and shoots or the rate of CK export from the roots is matched by shoot metabolism to maintain a steady-state level throughout the plant. These possibilities need to be explored further.

REFERENCES

- Taylor, J. S., M. Koshioka, R. P. Pharis, and G. B. Sweet. 1984. Changes in cytokinins and gibberellin-like substances in *Pinus radiata* buds during lateral shoot initiation and the characterization of ribosyl zeatin and a novel ribosyl zeatin glycoside. *Plant Physiology* 74: 626-631.

GENERAL DISCUSSION

Although it was not possible to exactly mimic episodic growth by inducing a surge in foliar growth, some aspects of shoot development were consistently enhanced by exogenous applications of CK. Treatment with BAP led to the precocious development of axillary meristems and the formation of adventitious buds. In addition, BAP treatment led to an underdeveloped root system. Taken together, these observations suggest that CKs may be involved in the control of episodic growth.

The developmental control exerted by PGRs is difficult to imitate using exogenous applications for at least three reasons. First, the potential fates of exogenously applied compounds (e.g., uptake, intra- and intercellular transport, localization, interconversion, and catabolism) are varied and poorly understood. Second, the route taken by exogenously applied PGRs may be different than that of their endogenously-produced equivalents. Finally, more than one group of PGR may be involved in controlling a given aspect of development (e.g., episodic growth). Nevertheless, red pine seedling development was consistently altered by exogenous applications of BAP.

Once it was shown that seedling development could be altered by CK treatment, the next logical step was to determine if endogenous CK levels change in advance of and in parallel with surges in shoot growth. Before performing these quantitative assessments, though, the endogenous CKs had to be qualitatively characterized. Initially this was done by detecting the presence of biological activity with the same chromatographic properties as authentic CK standards on both LH-20 and ODS-2. Although there were minor variations in the retention times of biological activity on the LH-20 column from run to run, the retention

times of the three major peaks of activity were constant relative to each other. Therefore, a ^3H -DHZ marker was added to each extract to locate the fractions containing biological activity without having to bioassay the LH-20 fractions from every run.

Including the ^3H -DHZ marker also provided a means of estimating the endogenous levels of CK in red pine. By knowing how much ^3H -DHZ was added to the extract and by measuring the amount of radioactivity present in each LH-20 fraction, the percent recovery at this step could be calculated. Using this information, in conjunction with the estimate of biological activity contained in each LH-20 fraction and the amount of tissue originally extracted, the level of endogenous CK could be expressed in terms of BAP equivalents. Accordingly, Z and ZR levels were repeatedly estimated to be less than 5 ng/g FW in both root and shoot tissue.

Due to these low levels and the presence of interfering compounds it was not possible to obtain mass spectra for all of the CKs produced by red pine. The fact that the compound responsible for biological activity at the retention time of ZR on both LH-20 and ODS-2 was indeed ZR lends some credibility to the other identifications. However, until they are confirmed using a more rigorous approach (e.g., GC-MS), the identities of these other compounds will have to remain tentative. Confirmation will not be accomplished by merely repeating the purification procedure using a larger extract. The impurities responsible for the high background levels seen in the GC-MS analysis were inseparable from CKs under the conditions used, so increasing the amount of tissue in the extract will only result in a proportionate increase in the level of contamination. Therefore, a way of removing the interference must be found.

Apart from ion exchange columns, the chromatographic systems used to isolate components of the pine extracts were reversed-phase in nature. Introduction of a different chromatographic system into the purification protocol may be necessary in order to remove enough interference to obtain mass spectra of the other CKs in red pine. Martin et al. (1981) developed a normal-phase HPLC procedure specifically for purifying permethyl CK derivatives. Scott and Horgan (1984) found this technique extremely useful as a final clean-up step for impure samples before subjecting them to mass spectrometric analysis. However, permethylated CKs are even more nonpolar than their underivatized counterparts and have low solubilities in the starting solvents used in normal-phase HPLC. This, combined with the manipulations required for an additional chromatographic purification step, would result in even lower recoveries than were achieved. Scott and Horgan (1984) were analyzing a tissue with an abundant supply of CK so any additional losses due to low solubility and handling were tolerable. Red pine, on the other hand, has extremely low levels of CK, so an additional HPLC step is not a preferred option.

Recently developed immunological techniques may provide a viable alternative to normal-phase HPLC. Many researchers are now using antibodies, raised against a specific PGR, in enzyme-linked- or radioimmunosorbent assays (ELISA and RIA, respectively) to quantify PGRs. Two types of antibodies are used for doing ELISAs and RIAs. The first, polyclonals, are a mixture of antibodies of an unknown titer and possessing a range of affinities and specificities. In contrast, monoclonals are generated by a single hybridoma cell line, which produces a monotypic supply of antibodies. All cell lines from a single fusion event are screened for their ability to produce antibodies with the highest possible specificity and affinity for a given structure. Monoclonal antibodies are much more

expensive to produce than polyclonal antibodies, so the latter are often used in place of the former.

The major disadvantage associated with these immunological techniques is that antibodies raised against a given PGR often show cross-reactivity to an array of chemically similar structures. Even with monoclonal antibodies, which have been screened carefully, cross-reactivity may be unavoidable, depending upon the uniqueness of the molecule against which the antibodies were raised. Due to the range of structurally similar CKs a given plant is capable of producing, cross-reactivity is inevitable when doing CK analyses. As a result, CKs must be separated chromatographically before being quantified using ELISA or RIA (MacDonald et al., 1981; Badenoch-Jones et al., 1984; Hansen et al., 1989).

Although cross-reactivity is a hindrance when performing ELISA or RIA, it can be advantageous when doing immunoaffinity chromatography. In fact, polyclonal antibodies are essential for doing certain types of purifications because of their range of specificities and affinities. Immunoaffinity chromatography involves cross-linking antibodies to a stable solid support (e.g., sepharose) to develop a packing material capable of removing structurally related compounds from a complex mixture. By including an immunoaffinity step early in the pine extract purification protocol, it may be possible to eliminate some of the other steps and still end up with a cleaner sample. Hopefully, immunoaffinity chromatography will allow for the removal of sufficient impurities from red pine extracts to complete the qualitative characterization of their endogenous CKs.

Once mass spectra have been obtained for each of the native CKs, their quantification can begin. During the quantification process, endogenous CKs are inevitably lost, and these losses vary from run to run. Therefore, an internal standard must be added when the tissue is extracted. This compound must behave,

chemically, like the endogenous CK being quantified but must be discernible at the final step in quantification. By knowing how much internal standard was added to the extract and the amount left after purification, the percent recovery can be calculated.

One factor that will influence the technique used to quantify the CKs (i.e., immunological vs physicochemical) is the availability of an internal standard. Scott and Horgan (1980) have described the use of ^{15}N -labelled CKs as internal standards for CK quantification. Because ^{15}N , a naturally-occurring, stable, heavy isotope of nitrogen is substituted for the lighter, more abundant, naturally-occurring isotope of nitrogen (^{14}N) at all four positions in the purine ring, the labelled compound is four atomic mass units heavier than its endogenous equivalent, and is easily distinguishable during mass spectrometry. These ^{15}N -labelled standards are difficult to synthesize and are not commercially available, so many researchers rely on commercially-available (Apex Organics Ltd., STEP Centre, Osney Mead, Oxford, OX2 0ES, U.K.), deuterated CK internal standards for doing GC-MS analysis.

However, neither the deuterated nor the ^{15}N -labelled CK internal standards can be used for immunological assays because antibodies cannot distinguish between them and the native CKs. To overcome this obstacle, some workers will split their sample in half, add a known quantity of a "cold" CK to one extract and purify this and an unenriched extract of the other half of the sample in parallel. By knowing how much more of this CK is present in the former than in the latter, after purification, the percent recovery can be calculated. Others will take a known quantity of cold standard alone through the entire purification procedure to obtain an estimate of recovery. Neither of these approaches is satisfactory because of the run-to-run variation in recovery. The only useful internal standards

for immunological approaches to CK quantification are radio-labelled (^{14}C and ^3H) varieties. By knowing the amount of radioactivity added to and recovered from the extract and the specific activity of the radio-labelled internal standard, recovery of the endogenous compound can be estimated.

No matter what internal standard is used, low concentrations must be added. Losses of PGRs result from incomplete partitioning, entrapment in other components, chemical reactions, or adsorption onto glassware or chromatographic supports. Absolute losses of PGRs due to oxidation, incomplete partitioning or entrapment will most likely be proportional to the amount of PGR in a sample over a wide range of concentrations. However, absolute losses due to adsorption are proportional to the amount of PGR in a sample only at low concentration. Once all the adsorption sites are filled, no additional PGR will be lost to adsorption. Therefore, internal standards must be added at concentrations similar to those normally found in the tissue in order to accurately account for losses due to adsorption (Brenner, 1981).

Two other factors that will influence the technique used to quantify endogenous CKs are the amount of CK present in the tissue and the level of contamination present after purification. If the level of CK is very low and the background levels are very high, the immunological approach may be best because it will only detect CK-like substances and it is sensitive in the picomolar range. In contrast, when doing GC-MS analysis, everything present in the sample gets fragmented and nanomolar quantities are required. Therefore, an immunoaffinity step may be needed before doing GC-MS analysis. Finally, it should be remembered that the immunological approach can only be taken after the endogenous CKs have been qualitatively characterized and separated chromatographically.

Using either of these approaches to CK quantification, it should be possible to determine if there is a correlation between levels of endogenous CKs and episodes of shoot growth. Although negative results from a correlative study would tend to rule out CK's involvement in the control of episodic growth, positive results would not necessarily provide a definitive answer. Because one event precedes another doesn't mean that the two have a cause and effect relationship; it could just be coincidence that CK levels increase prior to a surge in shoot growth. What is needed is a way to manipulate endogenous levels of CK and observe the effect. This may now be possible through genetic engineering.

It has recently been shown that a loblolly pine (*Pinus taeda*) is susceptible to infection by *Agrobacterium tumefaciens* (Sederoff et al., 1986). This provides a vehicle for inserting PGR biosynthetic genes into the pine genome. However, until a means of reliably regenerating plantlets from transformed pine cells becomes available, research in this area will be limited to gene expression at the cellular level. Transformation and regeneration have been accomplished using *Agrobacterium* on leaf discs in several dicotyledonous species (Horsch et al., 1985). This could serve as an alternative approach for recovering pine transformants until it is possible to clonally propagate pine from cultured cells.

It could be argued that the genetic engineering approach to answering the question of episodic growth is just another way of correlating endogenous levels of CK to surges in shoot growth and accomplishes the same thing as measuring CKs at various stages in seedling development. It is true that in both cases endogenous levels of CK are measured and a correlation is sought between these and a developmental response. However, the difference between the traditional correlative study and the genetic engineering approach is that the former is a passive process, whereas the latter is active. Rather than measure CK levels at

various stages in seedling development and hope for a relationship between endogenous levels of CKs and shoot activity, the endogenous CK levels could be deliberately altered in advance of an observable developmental response.

If a correlation between CK status and episodic growth can be established, the search for environmental cues that trigger a change in endogenous levels of CK can begin. This should eventually allow for the development of more effective cultural treatments for altering conifer seedling development.

LITERATURE CITED

- Akiyoshi, D. E., H. J. Klee, R. M. Amasino, E. W. Nester, and M. P. Gordon. 1984. T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. *Proceedings of the National Academy of Sciences USA* 81: 5994-5998.
- Akiyoshi, D. E., R. O. Morris, R. Hinz, B. S. Mischke, T. Kosuge, D. J. Garfinkel, M. P. Gordon, and E. W. Nester. 1983. Cytokinin/auxin balance in crown gall tumors is regulated by specific loci in the T-DNA. *Proceedings of the National Academy of Sciences USA* 80: 407-411.
- Alvim, R., E. W. Hewett, and P. F. Saunders. 1976. Seasonal variation in the hormone content of willow. I. Changes in abscisic acid content and cytokinin activity in the xylem sap. *Plant Physiology* 57: 474-476.
- Ashton, N. W., N. H. Grimsley, and D. J. Cove. 1979a. Analysis of gametophytic development in the moss *Physcomitrella patens*, using auxin and cytokinin resistant mutants. *Planta* 144: 427-435.
- Ashton, N. W., D. J. Cove, and D. R. Featherstone. 1979b. The isolation and physiological analysis of mutants of the moss, *Physcomitrella patens*, which over-produce gametophores. *Planta* 144: 437-442.
- Ashton, N. W., A. Schulze, P. Hall, and R. S. Bandurski. 1985. Estimation of indole-3-acetic acid in gametophytes of the moss, *Physcomitrella patens*. *Planta* 164: 142-144.
- Badenoch-Jones, J., D. S. Letham, C. W. Parker, and B. G. Rolfe. 1984. Quantitation of cytokinins in biological samples using antibodies against zeatin riboside. *Plant Physiology* 75: 1117-1125.
- Barry, G. F., S. G. Rogers, R. T. Fraley, and L. Brand. 1984. Identification of a cloned cytokinin biosynthetic gene. *Proceedings of the National Academy of Sciences USA* 81: 4776-4780.
- Brenner, M. L. 1981. Modern methods for plant growth substance analysis. *Annual Review of Plant Physiology* 32: 511-538.
- Buchmann, I., F.-J. Marner, G. Schroder, S. Waffenschmidt, and J. Schroder. 1985. Tumour genes in plants: T-DNA encoded cytokinin biosynthesis. *The EMBO Journal* 4(4): 853-859.
- Burdett, A. N. 1979. New methods for measuring root growth capacity: Their value in assessing lodgepole pine stock quality. *Canadian Journal of Forest Research* 9: 63-67.

- Burrows, W.J. and D. J. Carr. 1969. Effects of flooding the root system of sunflower plants on the cytokinin content in xylem sap. *Physiologia Plantarum* 22: 1105-1112.
- Chen, C.-m. 1982. Cytokinin biosynthesis in cell-free systems. Pages 155-163 in P. F. Wareing, ed. *Plant growth substances 1982*. Academic Press, New York.
- Clements, J. R. 1970. Shoot responses of young red pine to watering applied over two seasons. *Canadian Journal of Botany* 48: 75-80.
- Cove, D. J. and N. W. Ashton. 1984. The hormonal regulation of gametophytic development in bryophytes. Pages 177-201 in A. F. Dyer and J. G. Duckett, eds. *The experimental biology of bryophytes*. Academic Press, New York.
- Darimont, E., T. Gaspar, and M. Hofinger. 1971. Auxin-kinetin interaction on the lentil root growth in relation to indoleacrylic acid metabolism. *Zeitschrift für Pflanzenphysiologie* 64: 232-240.
- Doak, C. C. 1935. Evolution of foliar types, dwarf shoots, and cone scales of *Pinus*. *Illinois Biological Monographs* 13: 1-106.
- Drew, A. P. 1982. Shoot-root plasticity and episodic growth in red pine seedlings. *Annals of Botany* 49: 347-357.
- Drew, A. P. and F. T. Ledig. 1980. Episodic growth and relative shoot:root balance in loblolly pine seedlings. *Annals of Botany* 45: 143-148.
- Duff, G. H. and N. J. Nolan. 1958. Growth and morphogenesis in the Canadian forest species. III. The time scale of morphogenesis at the stem apex in *Pinus resinosa* Ait. *Canadian Journal of Botany* 36: 687-706.
- Duryea, M. L. 1984. Nursery cultural practices: Impacts on seedling quality. Pages 143-164 in M. L. Duryea and T. D. Landis, eds. *Forest nursery manual: Production of bareroot seedlings*. Martinus/Dr W. Junk Publishers, The Hague.
- Eggleston, K. L. and R. C. Sharp. 1986. Fertilizer trials on containerized red pine. Pages 38-42 in T. D. Landis and J. W. Fischer, eds. *Proceedings of the Intermountain Nurseryman's Association Meeting, Fort Collins, CO, August 13-15, 1985*. USDA Forest Service General Technical Report RM-125. 111 pp.
- Einset, J. W. and F. Skoog. 1973. Biosynthesis of cytokinins in cytokinin autotrophic tobacco callus. *Proceedings of the National Academy of Sciences USA* 70(3): 658-660.

- Engelbrecht, L. 1972. Cytokinins in leaf-cuttings of *Phaseolus vulgaris* L. during their development. *Biochemie und Physiologie der Pflanzen* 163: 335-343.
- Entsch, B., C. W. Parker, D. S. Letham, and R. E. Summons. 1979. Preparation and characterisation, using high-performance liquid chromatography, of an enzyme forming glucosides of cytokinins. *Biochimica et Biophysica Acta* 570: 124-139.
- Feldman, L. J. 1975. Cytokinins and quiescent center activity in roots of *Zea*. Pages 55-72 in J. G. Torrey and D. T. Clarkson, eds. *The development and function of roots*. Academic Press, New York.
- Garfinkel, D. J., R. B. Simpson, L. W. Ream, F. F. White, M. P. Gordon, and E. W. Nester. 1981. Genetic analysis of crown gall: Fine structure map of the T-DNA by site-directed mutagenesis. *Cell* 27: 143-153.
- Glerum, C. and G. Pierpoint. 1968. The influence of soil moisture deficits on seedling growth of three coniferous species. *The Forestry Chronicle* 44: 26-29.
- Goodwin, P. B. 1978. Phytohormones and growth and development of organs of the vegetative plant. Pages 31-173 in D. S. Letham, P. B. Goodwin and T. J. V. Higgins, eds. *Phytohormones and related compounds: A comprehensive treatise*. Vol. II. Elsevier/North-Holland Biomedical Press, The Netherlands.
- Granath, M. and C. Schuerch. 1953. Preliminary separations of maple hydrol lignin. *Journal of the American Chemical Society* 75: 707-709.
- Haggman, J. 1989. Cytokinins in the Scots pine buds during the spring. Abstract #291 presented at the 13th International Conference on Plant Growth Substances, Calgary, Canada, 17-22 July 1988.
- Hansen, C. E., C. Kopperud, and O. M. Heide. 1989. Immunoaffinity chromatography for purification of cytokinins in plant extracts. *Norwegian Journal of Agricultural Science* 3: 73-77.
- Holsters, R., R. Villarroel, J. Gielen, J. Seurinck, H. De Greve, M. Van Montagu, and J. Schell. 1983. An analysis of the boundaries of the octopine TL-DNA in tumors induced by *Agrobacterium tumefaciens*. *Molecular and General Genetics* 190: 35-41.
- Horgan, R. 1975. A new cytokinin metabolite. *Biochemical and Biophysical Research Communications* 65(1): 358-363.

- Horgan, R. 1984. Cytokinins. Pages 51-74 in M. B. Wilkins, ed. Advanced plant physiology. Longman Scientific and Technical, Harlow, Essex, England.
- Horgan, R. 1987. Cytokinin genes. Pages 119-130 in G. V. Hoad, J. R. Lenton, M. B. Jackson, and R. K. Atkin, eds. Hormone action in plant development: A critical appraisal. Butterworths, Cornwall, U. K.
- Horgan, R. and I. M. Scott. 1987. Cytokinins. Pages 303-365 in L. Rivier and A. Crozier, eds. Principles and practices of plant hormone analysis. Academic Press, New York.
- Horsch, R. B., J. E. Fry, N. L. Hoffmann, D. Eichholtz, S. G. Rogers, and R. T. Fraley. 1985. A simple and general method for transferring genes into plants. *Science* 227: 1229-1230.
- Iverson, R. D. 1984. Planting-stock selection: Meeting biological needs and operational realities. Pages 261-266 in M. L. Duryea and T. D. Landis, eds. Forest nursery manual: Production of bareroot seedlings. Martinus/Dr W. Junk Publishers, The Hague.
- Johri, M. M. 1974. Differentiation of caulonema cells by auxins in suspension cultures of *Funaria hygrometrica*. Pages 925-933 in Proceedings of the Eighth International Conference on Plant Growth Substances, Tokyo, Japan, August 26-September 1, 1973.
- Johri, M. M. 1975. The protonema of *Funaria hygrometrica* as a system for studying cell differentiation. Pages 116-124 in M. Y. Mohan Ram, J. J. Shah, and C. K. Shah, eds. Form and function in plants. Sarita Prakashan, Meerut, India.
- Johri, M. M. 1988. Development and differentiation in plants. *Bioscience Reports* 8(6): 553-564.
- Johri, M. M. and S. Desai. 1973. Auxin regulation of caulonema formation in moss protonema. *Nature* 245: 223-224.
- Key, J. L., J. Kimpel, and R. T. Nagao. 1987. Heat shock gene families of soybean and the regulation of their expression. Pages 87-97 in J. L. Key and L. McIntosh, eds. Plant gene systems and their biology. Alan R. Liss, Inc., New York.
- Klee, H. J., M. F. Yanofsky, and E. W. Nester. 1985. Vectors for transformation of higher plants. *Bio/Technology* 3: 637-642.
- Klee, H. J., R. Horsch, and S. Rogers. 1987a. *Agrobacterium*-mediated plant transformation and its further applications to plant biology. *Annual Review of Plant Physiology* 38: 467-486.

- Klee, H. J., R. Horsch, M. Hinchee, M. Hein, and N. Hoffman. 1987b. The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. *Genes and Development* 1: 86-96.
- Laloue, M. and C. Pethe. 1982. Dynamics of cytokinin metabolism in tobacco cells. Pages 185-195 in P. F. Wareing, ed. *Plant growth substances 1982*. Academic Press, London, U. K.
- Laloue, M., C. Terrine, and J. Guern. 1977. Cytokinins: metabolism and biological activity of N⁶-(Δ^2 -isopentenyl)adenine in tobacco cells and callus. *Plant Physiology* 59: 478-483.
- Lanner, R. M. 1976. Patterns of shoot development in *Pinus* and their relationship to growth potential. Pages 223-243 in M. G. R. Cannell and F. T. Last, eds. *Tree physiology and yield*.
- Ledig, T. T., A. P. Drew, and J. G. Clark. 1976. Maintenance and constructive respiration, photosynthesis, and net assimilation rate in seedlings of pitch pine (*Pinus rigida* Mill.). *Annals of Botany* 40: 289-300.
- Lee, T. S., J. G. Purse, R. J. Pryce, R. Horgan, and P. F. Wareing. 1981. Dihydroconiferyl alcohol--A cell division factor from *Acer* species. *Planta* 152: 571-577.
- Letham, D. S. 1978. Cytokinins. Page 205 in D. S. Letham, P. B. Goodwin, and T. J. V. Higgins, eds. *Phytohormones and related compounds: A comprehensive treatise*. Vol. I. Elsevier/North-Holland Biomedical Press, Amsterdam, The Netherlands.
- Letham, D. S., M. M. Wilson, C. W. Parker, I. D. Jenkins, J. K. MacLeod, and R. E. Summons. 1975. Regulators of cell division in plant tissues. XXIII. The identity of an unusual metabolite of 6-benzylaminopurine. *Biochimica et Biophysica Acta* 399: 61-70.
- Loewenberg, J. R. and F. Skoog. 1952. Pine tissue cultures. *Physiologia Plantarum* 5: 33-36.
- MacDonald, E. M. S. and R. O. Morris. 1985. Isolation of cytokinins by immunoaffinity chromatography and analysis by high-performance liquid chromatography radioimmunoassay. *Methods in Enzymology* 110: 347-358.
- MacDonald, E. M. S., D. E. Akiyoshi, and R. O. Morris. 1981. Combined high-performance liquid chromatography-radioimmunoassay for cytokinins. *Journal of Chromatography* 214: 101-109.

- Magnus, V., R. S. Bandurski, and A. Schulze. 1980. Synthesis of 4,5,6,7 and 2,4,5,6,7 deuterium-labeled indole-3-acetic acid for use in mass spectrometric assays. *Plant Physiology* 66: 775-781.
- Martin, G. C., R. Horgan, and I. M. Scott. 1981. High-performance liquid chromatographic analysis of permethylated cytokinins. *Journal of Chromatography* 219: 136-138.
- Mauney, J. R., W. S. Hillman, C. O. Miller, F. Skoog, R. A. Clayton, and F. M. Strong. 1952. Bioassay, purification, and properties of growth factor from coconut. *Physiologia Plantarum* 5: 485-497.
- McGaw, B. A. 1987. Cytokinin biosynthesis and metabolism. Pages 76-93 in P. J. Davies, ed. *Plant hormones and their role in plant growth and development*. Martinus Nijhoff, Dordrecht, The Netherlands.
- McGaw, B. A., R. Horgan, J. K. Heald, G. J. Wullens, and R. A. Schilperoort. 1988. Mass-spectrometric quantitation of cytokinins in tobacco crown-gall tumours induced by mutated octopine Ti plasmids of *Agrobacterium tumefaciens*. *Planta* 176: 230-234.
- Medford, J. I., R. Horgan, Z. El-Sawi, and H. J. Klee. 1989. Alteration of endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. *The Plant Cell* 1: 403-413.
- Miura, G. A. and R. H. Hall. 1973. Trans-ribosylation. Its biosynthesis in *Zea mays* endosperm and the mycorrhizal fungus *Rhizopogon roseolus*. *Plant Physiology* 51: 563-569.
- Mozes, R. and A. Altman. 1977. Characteristics of root-to-shoot transport of cytokinin 6-benzylaminopurine in intact seedlings of *Citrus aurantium*. *Physiologia Plantarum* 39: 225-232.
- Ooms, G., J. J. Hooykaas, G. Moolenaar, and R. A. Schilperoort. 1981. Crown gall tumors of abnormal morphology, induced by *Agrobacterium tumefaciens* carrying mutated octopine Ti plasmids; analysis of T-DNA functions. *Gene* 14: 33-50.
- Palmer, M. V., I. M. Scott, and R. Horgan. 1981. Cytokinin metabolism in *Phaseolus vulgaris* L. II. Comparative metabolism of exogenous cytokinins by detached leaves. *Plant Science Letters* 22: 187-195.
- Palni, L. M. S., L. Burch, and R. Horgan. 1988. The effect of auxin concentration on cytokinin stability and metabolism. *Planta* 174: 231-234.
- Parker, C. W. and D. S. Letham. 1974. Regulators of cell division in plant tissues. XVIII. Metabolism of zeatin in *Zea mays* seedlings. *Planta* 115: 337-

344.

- Parker, C. W., M. M. Wilson, D. S. Letham, D. E. Cowley, and J. K. MacLeod. 1973. The glycosylation of cytokinins. *Biochemical and Biophysical Research Communications* 55(4): 1370-1376.
- Parker, C. W., D. S. Letham, B. I. Gollnow, R. E. Summons, C. C. Duke, and J. K. MacLeod. 1978. Regulators of cell division in plant tissues. XXV. Metabolism of zeatin by lupin seedlings. *Planta* 142: 239-251.
- Paterson, J. M. 1987. Pine needle length in relation to field performance. Pages 85-91 in T. Marty, ed. *Proceedings of the Northeast Area Nurseryman's Conference*, Hayward State Nursery, Hayward WI, August 17-20, 1987.
- Paterson, J. M. and D. C. F. Fayle. 1984. Early prediction of plantation performance for red pine. *The Forestry Chronicle* 60(6): 340-344.
- Purse, J. G., R. Horgan, J. M. Horgan, and P. F. Wareing. 1976. Cytokinins in sycamore sap. *Planta* 132: 1-8.
- Qamaruddin, M. 1989a. The role of endogenous cytokinins in Scots pine (*Pinus sylvestris*) during transition from growth arrest to growth activity. Ph.D. dissertation. University of Stockholm, Stockholm, Sweden.
- Qamaruddin, M., I. Dormling, and L. Eliasson. 1989b. Increases in cytokinin levels in Scots pine in relation to chilling and bud burst. Submitted to *Physiologia Plantarum*.
- Radin, J. W. and R. S. Loomis. 1971. Changes in the cytokinins of radish roots during maturation. *Physiologia Plantarum* 25: 240-244.
- Rochester, D. E., J. A. Winer, and D. M. Shah. 1986. The structure and expression of maize genes encoding the major heat shock protein, hsp 70. *The EMBO Journal* 5(3): 451-458.
- Rogozinska, J. H. 1967. The occurrence of cytokinins in Scots pine. *Bulletin de L'Academie Polonaise des Sciences* 15(12): 789-794.
- Rogozinska, J. H., J. P. Helgeson, F. Skoog, S. H. Lipton, and F. M. Strong. 1965. Partial purification of cell-division factor from peas. *Plant Physiology* 40: 469-476.
- Schroder, G., S. Waffenschmidt, E. W. Weiler, and J. Schroder. 1984. The T-region of Ti plasmid codes for an enzyme synthesizing indole-3-acetic acid. *European Journal of Biochemistry* 138: 387-391.

- Scott, I. M. and R. Horgan. 1980. Quantification of cytokinins by selected ion monitoring using ^{15}N -labelled internal standards. *Biomedical Mass Spectrometry* 7: 446-449.
- Scott, I. M. and R. Horgan. 1984. Mass spectrometric quantification of cytokinin nucleotides and glycosides in tobacco crown gall tissue. *Planta* 161: 345-354.
- Scott, T. K. 1972. Auxins and roots. *Annual Review of Plant Physiology* 23: 235-258.
- Sederoff, R., A.-M. Stomp, W. S. Chilton and L. W. Moore. 1986. Gene transfer into loblolly pine by *Agrobacterium tumefaciens*. *Bio/Technology* 4: 647-649.
- Sembdner, G. and D. Gross. 1986. Plant growth substances of plant and microbial origin. Pages 139-147 in M. Bopp, ed. *Plant growth substances 1985*. Springer-Verlag, Berlin.
- Short, K. C., J. G. Torrey. 1972. Cytokinins in seedling roots of pea. *Plant Physiology* 49: 155-160.
- Skene, K. G. M. 1975. Cytokinin production by roots as a factor in the control of plant growth. Pages 365-396 in J. G. Torrey and D. T. Clarkson, eds. *The development and function of roots*. Academic Press, New York.
- Skoog, F. and C. O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured *in vitro*. *Symposia of the Society for Experimental Biology* 11: 118-140.
- Sondheimer, E. and D.-S. Tzou. 1971. The metabolism of hormones during seed germination and dormancy. II. The metabolism of $8\text{-}^{14}\text{C}$ -zeatin in bean axes. *Plant Physiology* 47: 516-520.
- Stone, E. C. 1955. Poor survival and the physiological condition of planting stock. *Forest Science* 1: 90-94.
- Strothmann, R. O. 1967. The influence of light and moisture on the growth of red pine seedlings in Minnesota. *Forest Science* 13: 182-191.
- Sucoff, E. 1971. Timing and rate of bud formation in *Pinus resinosa*. *Canadian Journal of Botany* 49: 1821-1832.
- Svensson, S.-B. 1972. A comparative study of the changes in root growth induced by coumarin, auxin, ethylene, kinetin and gibberellic acid. *Physiologia Plantarum* 26: 115-135.
- Taylor, J. S., M. Koshioka, R. P. Pharis, and G. B. Sweet. 1984. Changes in cytokinins and gibberellin-like substances in *Pinus radiata* buds during lateral

- shoot initiation and the characterization of ribosyl zeatin and a novel ribosyl zeatin glycoside. *Plant Physiology* 74: 626-631.
- Trewavas, A. 1983. Is plant development regulated by changes in the concentration of growth substances or by changes in the sensitivity to growth substances? *Trends in Biochemical Sciences* 8(10): 354-357.
- van den Driessche, R. 1983. Growth, survival, and physiology of Douglas-fir seedlings following root wrenching and fertilization. *Canadian Journal of Forest Research* 13: 270-278.
- Van Onckelen, H., P. Rudelsheim, D. Inze, A. Follin, E. Messens, S. Horemans, J. Schell, M. Van Montagu, and J. De Greef. 1985. Tobacco plants transformed with the *Agrobacterium* T-DNA gene 1 contain high amounts of indole-3-acetamide. *Federation of European Biochemical Societies Letters* 181: 373-376.
- Van Onckelen, H., E. Prisen, D. Inze, P. Rudelsheim, M. Van Lijsebettens, A. Follin, J. Schell, M. Van Montagu, and J. De Greef. 1986. *Agrobacterium* T-DNA gene 1 codes for tryptophan 2-monooxygenase activity in tobacco crown gall cells. *Federation of European Biochemical Societies Letters* 198: 357-360.
- Van Staden, J. and J. E. Davey. 1979. The synthesis, transport and metabolism of endogenous cytokinins. *Plant, Cell and Environment* 2: 93-106.
- Von Schwartzberg, K., K. Lutze, and H. Hahn. 1988. Determination of cytokinins in needles of spruce (*Picea abies* (L.) Karst.) by an indirect enzyme-linked immunosorbent assay. *Journal of Plant Physiology* 133: 529-534.
- Wang, K., L. Herrera-Estrella, M. Van Montagu, and P. Zambryski. 1984. Right 25 bp terminus sequence of the nopaline T-DNA is essential for and determines direction of DNA transfer from *Agrobacterium* to the plant genome. *Cell* 38: 455-462.
- Wang, T. L., R. Horgan, and D. J. Cove. 1981a. Cytokinins from the moss *Physcomitrella patens*. *Plant Physiology* 68: 735-738.
- Wang, T. L., P. Beutelmann, and D. J. Cove. 1981b. Cytokinin biosynthesis in mutants of the moss, *Physcomitrella patens*. *Phytochemistry* 19: 1103-1105.
- Weiler, E. W. 1980. Radioimmunoassay for trans-zeatin and related cytokinins. *Planta* 135: 285-288.
- Weiler, E. W. and K. Spanier. 1981. Phytohormones in the formation of crown gall tumors. *Planta* 153: 326-337.

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APPENDIX A: HISTOLOGICAL ANALYSIS OF BUD CLUSTERS INDUCED ON RED PINE SEEDLINGS THROUGH EXOGENOUS APPLICATIONS OF BENZYLAMINOPURINE

Introduction

There are numerous reports of *in vitro* adventitious bud induction on various explant sources through exogenous applications of cytokinin (see von Arnold and Eriksson, 1980 and Horgan and Aitken, 1981 for references). There are also a few reports of *in vivo* bud formation in response to exogenous applications of cytokinin (Kossuth, 1978; Little, 1984, 1985; Mulgrew and Williams, 1985; Mazzola and Costante, 1987; Zel et al., 1988). The latter treatment is often applied to whole trees to stimulate lateral bud development. Removal of the terminal bud on these pretreated trees leads to release from apical dominance, causing the newly-formed and preexisting lateral buds to flush. The result is a tree with greater foliar density, a very desirable characteristic for Christmas tree growers.

In a previous paper (see Section II), we described the response of red pine (*Pinus resinosa* Ait.) seedlings to exogenous applications of benzylaminopurine (BAP). This treatment was intended to induce a surge of shoot activity in order to demonstrate cytokinin's involvement in the control of episodic growth (Drew, 1982). Rather than increase foliar production, these treatments led to the formation of a terminal cluster of buds. The number of buds formed was in excess of the number of leaf axils present on the seedlings receiving treatment. In addition, Lester (1968) has shown that not all primary-leaf axils contain meristems. Because an adventitious structure is defined as one which "arises at an unusual site" (Esau, 1977), induced buds could be both axillary and adventitious (i.e., in this case, buds which are not axillary must be adventitious).

Histological analysis was conducted on BAP-treated and untreated red pine seedlings to identify the origin of these buds. The results of this work are reported here.

Materials and Methods

Culture conditions for the greenhouse-grown red pine seedlings have already been described in Section I. Eleven days after sowing, biweekly BAP treatments were begun. An aqueous solution containing 25 mg/l BAP and 0.075% (v/v) Tween 20 was applied using the foliar dip technique described in Section II. After each treatment, seedlings were not watered for at least 12 hours to avoid washing away the BAP solution. Initially there were 60 seedlings in the treatment group and 36 control seedlings.

Five treated seedlings and three control seedlings were harvested on each of 12 successive days and prepared for histological analysis. Harvesting was done at the same time each day; on treatment days, seedlings were harvested prior to the BAP application. Harvesting involved severing the hypocotyl one centimeter below the epicotyl and trimming all needles to the same length with a single-edged razor.

Standard paraffin methods, adapted from Jensen (1962) and Berlyn and Miksche (1976), were used for the preparation of microscope slides. Tissue samples were fixed in FAA (formalin:acetic acid:ethyl alcohol, 1:1:18), dehydrated through a tertiary butyl alcohol series, embedded in Paraplast (VWR Scientific, P.O. Box 66929, O'Hare AMF, Chicago, IL, 60666), and sectioned longitudinally on a rotary microtome at a thickness of 10 μ m. These sections were affixed to slides with Haupt's adhesive, differentially stained with safranin and fast green, and mounted under a #1 coverslip with Permount (Fisher Scientific, 1600 W).

Glenlake Ave., Itasca, IL, 60143). Slides were viewed and photographed through an Wild microscope (model M7A).

Results

Cytokinin treatment resulted in the formation of buds in nearly every leaf axil (Fig. 1A), whereas axillary buds were absent on control seedlings of about the same age (Fig. 1B). At higher magnification, it appears that buds are forming within the axils of other buds, both of which presumably are produced in response to BAP treatment (Fig. 1C). Structures similar to those shown in Fig. 1D were frequently seen in treated seedlings.

Discussion

Although axillary meristems are usually absent from 18-day-old red pine seedlings, they do eventually form in some of the primary-leaf axils (Lester, 1968). Therefore, BAP treatment appears to stimulate precocious axillary bud development. Because axillary meristems do not ordinarily form within the axils of other meristems, it would appear that BAP treatment is also stimulating the formation of adventitious buds. However, this histological analysis was conducted too early in the developmental sequence, so this latter conclusion will have to remain tentative until fully developed adventitious structures are observed.

The structures seen throughout the apices of treated seedlings (Fig. 1D) resemble two vascular bundles, side-by-side. Because the induced buds formed after the needles with which they are associated, they may require a separate vascular system. This may explain this unusual juxtaposition of vascular bundles (Lersten, 1988). In order to resolve this question fully, a detailed analysis of

Figure 1. Cross-sections of red pine shoot apices

- (A) From a seedling treated with 25 mg/l BAP. The tissue was fixed when the seedling was 18 days old and had received 2 BAP treatments. Arrows indicate the location of meristems (40X)**
- (B) From a 23-day-old control seedling (40X)**
- (C) Magnification of the region in the lower right-hand corner of (A). Arrows indicate what appear to be separate meristems (100X)**
- (D) Vascular-like structures frequently found in cross-sections of BAP-treated apices (100X)**



the vascular development in association with normal axillary buds must be undertaken.

Literature Cited

- Berlyn, G. P. and J. P. Miksche. 1976. Botanical microtechnique and cytochemistry. Iowa State University Press, Ames, Iowa, 50011.
- Drew, A. P. 1982. Shoot-root plasticity and episodic growth in red pine seedlings. *Annals of Botany* 49: 347-357.
- Esau, K. 1977. Anatomy of seed plants. John Wiley and Sons, New York.
- Horgan, K. and J. Aitken. 1981. Reliable plantlet formation from embryos and seedling shoot tips of radiata pine. *Physiologia Plantarum* 53: 170-175.
- Jensen, W. A. 1962. Botanical histochemistry. W. H. Freeman and Co., San Francisco, CA.
- Kossuth, S. V. 1978. Induction of fascicular bud development in *Pinus sylvestris* L. *HortScience* 13: 174-176.
- Lersten, N. R. 1988. Personal communication. Anatomist, Department of Botany, Iowa State University, Ames, Iowa, 50011.
- Lester, D. T. 1968. Developmental patterns of axillary meristematic activity in seedlings of *Pinus*. *Botanical Gazette* 129(3): 206-210.
- Little, C. H. A. 1984. Promoting bud development in balsam fir Christmas trees with 6-benzylaminopurine. *Canadian Journal of Forest Research* 14: 447-451.
- Little, C. H. A. 1985. Increasing lateral shoot production in balsam fir Christmas trees with cytokinin application. *HortScience* 20(4): 713-714.
- Mazzola, M. and J. F. Costante. 1987. Efficacy of BA for the promotion of lateral bud formation on Douglas-fir and Colorado blue spruce. *HortScience* 22(2): 234-235.
- Mulgrew, S. M. and D. J. Williams. 1985. Effect of benzyladenine on the promotion of bud development and branching of *Picea pungens*. *HortScience* 20: 380-381.
- von Arnold, S. and T. Eriksson. 1980. *In vitro* studies of adventitious shoot formation in *Pinus contorta*. *Canadian Journal of Botany* 59: 870-874.

Zel, J., N. Gogala, and M. Camloh. 1988. Micropropagation of *Pinus sylvestris*.
Plant Cell, Tissue and Organ Culture 14: 169-175.

APPENDIX B: TOWARD A CLONAL PROPAGATION SYSTEM FOR RED PINE

Introduction

The apical cluster of adventitious buds produced in response to exogenous applications of benzylaminopurine (BAP; see Section II) may provide an novel approach to micropropagating red pine. If the buds could be induced to elongate, it might be possible to separate and root the resulting shoots. However, because the bud clusters formed on seedlings that were grown in a greenhouse, a surface sterilization technique needed to be developed before the buds could be cultured *in vitro*. As an alternative to micropropagation using the bud clusters, mature embryos were extracted from surface-sterilized red pine seed in an attempt to clonally propagate red pine through organogenesis.

Materials and Methods

Micropropagation using terminal bud clusters

Seedling culture conditions were specified in Section I. Three-week-old seedlings were treated with 200 mg/l BAP using the foliar dip technique outlined in Section II. The resulting bud clusters were excised from 14-week-old seedlings, stirred in a 15% solution of commercial bleach for 15 minutes, and rinsed one time with 70% ethyl alcohol and three times with sterile, distilled water. The duration of each of these rinses was about 30 seconds. The bud clusters were then cultured aseptically on Murashige and Skoog (M-S; 1962) media containing various combinations of auxin and cytokinin. Four levels each of BAP (10, 1, 0.1 and 0 μ M) and naphthalene acetic acid (NAA; 1, 0.1 and 0.01, and 0 μ M) were tested in a complete factorial design. Clusters containing buds which elongated

were subcultured on basal M-S media.

Clonal propagation via organogenesis

Red pine seeds were surface-sterilized using the procedure just described for the bud clusters. Following surface-sterilization, the seed coat was still too hard for convenient embryo extraction, so the seed was imbibed for three days to soften the seed coat. Embryos extracted from seed that had been surface-sterilized and imbibed showed severe contamination once in culture. Therefore, the moistened seed had to be surface-sterilized a second time before embryo extraction. After three days of imbibition on autoclaved blotter paper saturated with sterile, distilled water, the seeds were stirred in a 10% solution of bleach for 10 minutes, followed once again by one rinse with 70% ethyl alcohol and three rinses with sterile, distilled water.

Embryos were extracted in a laminar flow hood. This was done by slicing the seed longitudinally, slightly off center to avoid damaging the embryo, and digging through the endosperm with a dissection needle. The exposed embryos were lifted out of the endosperm with sterile forceps and placed on M-S media. The same complete factorial design used in the micropropagation study was also used to test the effect of cytokinin and/or auxin on organogenesis. Following the appearance of adventitious shoots on the callus derived from these excised embryos, the calli were subcultured on basal M-S media. Cultures were maintained under continuous fluorescent light (Philips, VHO, cool white) at 25°C.

Results

Micropropagation using terminal bud clusters

When bud clusters were plated on basal media, none of the buds grew out (Figure 1A). A few of the buds elongated when clusters were cultured on M-S

Figure 1. Surface-sterilized bud clusters induced on red pine seedlings through foliar applications of 200 mg/l BAP. These bud clusters were all ca. 1 cm in diameter and were plated on M-S media containing:

(A) No PGRs

(B) 1.0 μ M BAP + 0.1 μ M NAA

(C) 1.0 μ M BAP + 1.0 μ M NAA



media containing 1.0 μM BAP + 0.1 μM NAA (Figure 1B), whereas most of the buds elongated when a cluster was plated on M-S media containing 1.0 μM BAP + 1.0 μM NAA (Figure 1C). Unfortunately, the shoots did not develop further and, consequently, were never rooted.

Clonal propagation via organogenesis

Two weeks after extraction, embryos plated on basal media, media containing the two lowest levels of NAA (0.1 and 0.01 μM), and media containing a combination of 0.1 μM BAP + 0.01 μM NAA all looked similar to the one shown in Figure 2A. The cotyledons are a bright green color and the hypocotyl was red. Regardless of the level used, all embryos plated on media containing only BAP were similar in appearance to the one depicted in Figure 2B. Some green translucent callus was formed but no organogenesis took place. All embryos plated on media containing the highest level of NAA (1.0 μM), whether alone or in combination with BAP, turned a white color and died (Figure 2C).

When embryos were plated on media containing either 1.0 μM BAP + 0.1 μM NAA or 1.0 μM BAP + 1.0 μM NAA, adventitious shoots were initiated in the callus derived from the embryonic explants (Figure 2D). When this callus was subcultured on basal media, shoot development occurred (Figure 2E). However, the callus and shoots eventually turned a brown color and died (Figure 2F). Although the callus was repeatedly subcultured on basal media, some of which contained activated charcoal, this degenerative process could not be prevented.

Discussion

Micropropagation using terminal bud clusters

While it was not possible to produce rooted seedlings from the bud clusters cultured *in vitro*, this technique may still show some promise as a means of mi-

Figure 2. Tissue cultures derived from excised red pine embryos plated on M-S media containing the specified supplements. Unless otherwise indicated, the cultures were 2 weeks old when the photos were taken. All photos were taken at the same magnification; the length of the callus in (B) is ca. 1 cm

(A) An embryo plated on basal media

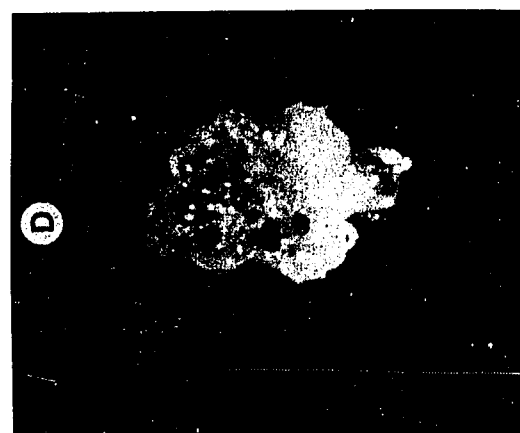
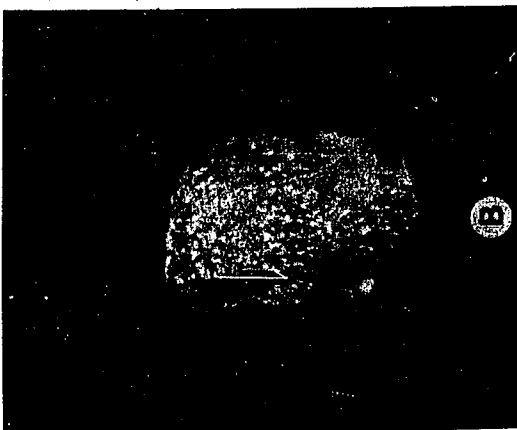
(B) Callus derived from an embryo plated on media containing 1.0 μM BAP

(C) Callus derived from an embryo plated on media containing 1.0 μM NAA

(D) Callus with adventitious shoots derived from an embryo plated on media containing 1.0 μM BAP + 0.1 μM NAA

(E) The culture from (D) subcultured on basal media at 4.5 weeks and allowed to develop for an additional 1.5 weeks

(F) A ten-week-old culture which had been subcultured on basal media 2 times, at one month intervals. The final medium contained activated charcoal (5 g/l)



cropropagating red pine. In a recent article by Zel et al. (1988), a very similar approach was taken with *Pinus sylvestris*. However, these authors were able to recover elongated shoots for rooting by: 1) separating the buds when they were about one millimeter long, 2) providing the buds with a pulse of BAP (111 μ M for two hours), 3) plating the buds on media (M-S) containing reduced levels of sucrose (2% instead of the usual 3%), and 4) exposing the buds to far-red light (690 to 720 nm) for eight hours immediately after plating. In light of their findings, it may prove fruitful to repeat the procedure of Zel et al. using red pine.

Clonal propagation via organogenesis

Based on the work of Gupta and Durzan (1987), it would appear that at least two modifications need to be made in order to successfully propagate red pine via embryo explants. The first concerns the interval between subculturings. Cell browning followed by death of the callus are widespread occurrences in pine tissue culture. These authors were able to avoid this outcome, and maintain their callus cultures for over one year, by subculturing every 10-12 days. This is a much shorter interval than was used in the present study (about 30 days).

The second problem concerns the nature and developmental stage of the explant source. Gupta and Durzan (1986) were able to repeatedly induce somatic polyembryogenesis (as opposed to organogenesis) from a "proliferating embryonal-suspensor mass" derived from seeds harvested about four weeks after fertilization in *P. lambertiana* and in *P. taeda* (Gupta and Durzan, 1987). The development time and morphology of these induced embryos were very similar to those found in the early stages of zygotic embryogenesis. When the embryos derived from embryonal-suspensor masses were grown on basal media without supplements and with 0.25% (w/v) activated charcoal under continuous light, complete

plantlets were formed. However, these authors had very little success when using suspensor cells or embryos derived from mature seeds or when using immature female gametophyte tissue that was not attached to suspensor cells. Therefore, in future attempts to clonally propagate red pine, the use of immature embryonal-suspensor masses should be strongly considered.

Literature Cited

- Gupta, P. K. and D. J. Durzan. 1986. Somatic polyembryogenesis from callus of mature sugar pine embryos. *Bio/Technology* 4: 643-645.
- Gupta, P. K. and D. J. Durzan. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. *Bio/Technology* 5: 147-151.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.
- Zel, J., N. Gogala, and M. Camloh. 1988. Micropropagation of *Pinus sylvestris*. *Plant Cell, Tissue and Organ Culture* 14: 169-175.

APPENDIX C: THE RELATIONSHIP BETWEEN FRESH AND DRY WEIGHTS

Introduction

Plant growth regulator content is most often expressed on a per gram fresh weight basis. However, the red pine tissue that was used for the qualitative cytokinin determinations had to be freeze-dried for transport to Aberystwyth, Wales, where the analyses were conducted. In order to convert the dry weight of tissue being extracted to its fresh weight equivalent, a relationship between fresh weight (FW) and dry weight (DW) had to be established. Because root and shoot material were to be extracted separately, a FW/DW quotient was needed for each tissue type.

Procedure

Three groups of seedlings were harvested on three separate dates. The seedlings harvested on 6/29/88 were planted on 2/1/88, those harvested on 8/1/88 and 9/16/88 were planted on 2/19/88 and 5/19/88, respectively. Harvests were performed by removing seedlings from their containers, shaking away as much potting mix as possible, rinsing the roots with tap water, separating the roots from the shoots by severing the stem one centimeter above the first lateral root and shaking away the excess water.

The material to be taken to Aberystwyth had to be frozen immediately, in order to preserve its physiological state at the time of harvest, so fresh weight determinations could not be made on this material. Instead, a five to ten percent sample of the seedlings from each harvest were randomly selected to obtain estimates of shoot and root FWs. After shaking away the excess water, roots and shoots from these seedlings were dabbed with a dry paper towel and allowed to

air-dry for three to five minutes before weighing. These seedlings were then discarded. Their average root and shoot FWs were multiplied by the number of seedlings in each harvest, to obtain an estimate of total root and shoot FWs.

The plant material from each harvest that was to be taken to Aberystwyth was immediately frozen in liquid nitrogen and then stored at -70°C until freeze-drying, after which it was weighed *in toto*. The total root and shoot FWs for each harvest were divided by their corresponding DWs to obtain three FW/DW quotients, which were then averaged.

Results

Fresh and dry weight data for the roots and shoots and the number of seedlings contributing to these estimates in each harvest are listed in Table 1.

Discussion

Provided that the freeze-dried tissue is weighed prior to extraction, the average FW/DW ratios appearing in Table 1 can be used to express the hormone content of each extract on a per gram fresh weight basis.

Table 1. Average fresh and dry weight data, by harvest date, of four- and seven-month-old red pine seedlings

TT-HD ^a	Avg. FW ^b	Tot. FW ^c	Tot. DW ^d	FW/DW	Wtd. Avg. FW/DW ^e
S-1	3.21 (12, 0.117)	832.2	79.9	10.4	7.8
S-2	3.47 (14, 0.267)	909.1	132.6	6.9	
S-3	0.52 (27, 0.038)	150.8	21.1	7.1	
R-1	2.22 (12, 0.073)	574.7	196.6	2.9	2.4
R-2	2.65 (14, 0.219)	694.3	269.5	2.6	
R-3	0.35 (27, 0.023)	101.5	48.6	2.1	

^aTT=tissue type (S=shoots, R=roots); HD=harvest date (1=6/29, 2=8/1, 3=9/16/88).

^bAverage root and shoot fresh weights. Based on a 5% sample. The numbers in parentheses are the sample size and standard error of the mean, respectively (n, SEM).

^cTot. FW=estimated total fresh weight of the material from each harvest that was freeze-dried. Obtained by multiplying the averaged FW of roots and shoots, from the 5% sample, by the number of seedlings that contributed to the DW estimates (259, 276 and 290 seedlings for dates 1-3, respectively).

^dTot. DW=total dry weight of all material from each harvest that was freeze-dried.

^eWtd. Avg. FW/DW=weighted average ratio for all three harvest dates.

APPENDIX D: REVISION OF THE *AMARANTHUS* ASSAY

Background

Because of the speed with which the *Amaranthus* assay can be conducted, it continues to be very popular for detecting and quantifying cytokinins (CKs) in plant extracts enroute to more exacting chemical analyses. However, the published protocol for this assay (Biddington and Thomas, 1973) lacks the sensitivity desired in work of this kind. All bioassays require destructive sampling; the portion of a fraction that is assayed cannot be recovered for further purification and analysis. By improving the sensitivity of this assay, less sample needs to be sacrificed and more is available for further characterization of the biological activity. Utilizing less sample per assay leads to a reduction in the amount of tissue needed and a substantial savings in time and supplies.

In order to achieve this improvement in sensitivity, several changes have been made in the published protocol. The first concerns the volume of distilled water (dH₂O) used for extracting the cotyledons. Biddington and Thomas recommended using three milliliters (ml). Because a given amount of pigment will absorb more light when it is concentrated, a lower absolute amount of pigment can be detected in a smaller volume of water. With this in mind, the cotyledons were placed in 800 microliters (μl) dH₂O for freezing and thawing rather than the recommended volume. Biddington and Thomas also recommended two freeze/thaw cycles to rupture cell membranes and release the water-soluble pigment. Because residual pigment was observed in cotyledons that had undergone three freeze/thaw cycles, the cotyledons were extracted using five freeze/thaw cycles.

Once the pigment extraction was completed, Biddington and Thomas recommended measuring the optical density of the extract at 542 and 620 nanometers (nm). The former is a wavelength of light absorbed by the pigment, the latter is not. The reading taken at the second wavelength is subtracted from the first in order to compensate for suspended debris which reflects, absorbs and scatters light. Although it was not suggested by Biddington and Thomas, extracts were centrifuged at 11,600 g for 10 min. before making absorbance determinations to remove this source of interference.

To determine the wavelength of maximum absorption by the pigment, numerous extracts were scanned over the entire visible range of the electromagnetic spectrum. Each time, the wavelength of maximum absorption was determined to be 535 nm! Because the range of wavelengths in which the pigment absorbs is narrow, taking absorbance readings seven nm away from the maximum has a dramatic affect on the outcome. Therefore, optical density measurements were taken at 535 and 620 nm in all the bioassays performed during the work described herein. This work was subsequently repeated in another lab, using a different spectrophotometer and a different source of *Amaranthus caudatus* seed. Once again, the wavelength of maximum absorbance was 535 nm. This confirms that the spectrophotometer used in the original work was properly calibrated.

Finally, assays were performed in five-centimeter (cm), glass Petri dishes. Even though the dishes were thoroughly washed with detergent and rinsed with tap water and dH₂O between assays, there was a marked decline in sensitivity when the same dish was used repeatedly. It was thought that an inhibitor present in the plant extracts was adsorbing to the glass and was gradually released during the incubation phase of the assay. To prevent this inhibition, the Petri dishes were soaked overnight in chromic acid and rinsed with tap water before

being washed in the usual way.

Revised Protocol

Sowing

Seed (0.70 g is sufficient for ca. 32 assays) was aligned in rows on one layer of Whatman 3MM chromatography paper (ca. 15 X 25 cm), saturated with glass-dH₂O, in a plastic seed tray with the holes in the bottom taped. A little excess dH₂O was left in the tray to compensate for evaporation and imbibition. The tray was covered with aluminum foil, wrapped in a black plastic bag and stored at 25°C for 96 hr.

Sample transfer

Samples to be assayed were dissolved in methanol (MeOH) and transferred to chromic acid-cleaned, numbered, glass Petri dishes containing one 4.25-cm Whatman no. 1 filter-paper disc. Unused discs were sealed in an air-tight container. The MeOH was allowed to evaporate off in a fume hood. Plates were covered with a wide mesh screen, thus allowing the MeOH to evaporate but prevent the filter discs from blowing away.

Preparation of BAP standards

Using a 1 µg/µl N⁶-benzylaminopurine (BAP) stock solution in ethanol (EtOH), a 1 X 10⁻⁵ M working solution was prepared. This was diluted, serially, to arrive at the following concentrations: 1 X 10⁻⁶, 5 X 10⁻⁷, 1 X 10⁻⁷, 5 X 10⁻⁸ and 1 X 10⁻⁸ M. One ml of each standard and the zero controls (only EtOH) were pipetted into replicate plates lined with Whatman no. 1 filter paper, and dried as described above.

Preparation of phosphate buffer

To the desired volume of 2.1 g/l NaH_2PO_4 , 1.9 g/l Na_2HPO_4 was added dropwise until pH 6.3 was achieved. This was added to sufficient L-tyrosine to achieve a final concentration of 1 mg/ml. The flask containing the buffer was covered with aluminum foil and heated, swirling occasionally, until L-tyrosine was dissolved. The buffer was then allowed to cool to room temperature.

Samples/standards dissolved in buffer

After evaporation of the alcohol from sample and standard plates, 1.0 ml of buffer was added to each dish. (N.B. After covering the dish with its lid, it was tilted and swirled gently to rinse the sides of the plate because the MeOH and EtOH adhered to the side of the dish and some sample/standard could be left on the sides of the dish.)

Cotyledons assigned to dishes

Ten sets of 96-hour-old cotyledons were systematically assigned to each plate, one at a time. This was done in a very dimly lit room. The seeds were sown in rows to easily select a group of seedlings at one time with forceps. These were laid on a clean piece of glass. The upper one cm of the seedlings (cotyledons) was cut from the entire group simultaneously with a clean, single-edged razor blade. Cotyledons were transferred to plates, one set at a time, in series, to avoid bias. (One tends to select the larger seedlings first and there may be some variability in betacyanin production between size classes.) After there was one set of cotyledons per dish, the process was repeated until there were 10 sets of cotyledons per dish.

Incubation

The 3MM paper and unused seedlings were removed from the tray and the excess dH₂O shaken out. The dishes were placed in the tray, covered with aluminum foil, inserted in a black plastic bag, and stored at 25°C for 18 hr.

Pigment extraction

Eight hundred microliters (or less, depending on the size of the cuvette to be used for spectrophotometric readings--the less the better) of dH₂O was added to enough Eppendorf tubes to have one for each dish. With forceps, the cotyledons were transferred from each plate to a correspondingly numbered Eppendorf tube, making sure the cotyledons were completely submerged. The Eppendorf tubes were placed in an aluminum tray with dividers, and transferred between a glassware-drying oven (ca. 60°C) and a -20°C chest freezer every 30 min. After five freeze/thaw cycles, the Eppendorf tubes were shaken vigorously for about 10 seconds and centrifuged at 11,600 g for 10 min.

Spectrophotometry

The supernatants were added to a small-volume (600 ul) quartz cuvette and read at 535 nm and 642 nm in a spectrophotometer, in the dual wavelength mode, so as to record the difference in absorbance (ΔA) at the two wavelengths.

Results

Each time a plant sample was bioassayed, a duplicated set of BAP standards was also assayed. The same BAP concentrations were used each time: 1.30×10^{-8} , 6.52×10^{-8} , 1.30×10^{-7} , 6.52×10^{-7} , and 1.30×10^{-6} M. In addition, a zero control was tested each time. The average response for 10 such assays is plotted

in Figure 1.

At one point it was thought that red pine contained zeatin 7-glucoside (Z7G). Although Letham et al. (1983) have tested the sensitivity of the *Amaranthus* assay to various glucosides, Z7G was not included in their trials. Therefore, a series of Z7G standards (1×10^{-8} , 1×10^{-7} and 1×10^{-6} M) was assayed in parallel with the usual BAP standards. The results of this work are also shown in Figure 1, where they are plotted next to their closest BAP equivalent (concentration).

Discussion

Using the protocol of Biddington and Thomas (1973), the minimum detectable concentration of kinetin is 5×10^{-6} molar (M; Horgan, 1984). Employing the revised protocol outlined above, it was possible to detect $1-5 \times 10^{-8}$ M BAP. The sensitivity of the *Amaranthus* assay to kinetin is probably not equivalent to that of BAP, so a direct comparison is not possible. However, the response of zeatin is comparable to that of BAP and LaMotte (1990) has shown (unpublished data) that, using the Biddington and Thomas (1973) protocol, the *Amaranthus* assay is capable of detecting 10^{-7} M zeatin. Therefore, it may be possible to detect as little as 10^{-9} M zeatin using the modified procedure.

Further improvements in sensitivity of the assay can be achieved by 1) extending the incubation time, 2) increasing the number of cotyledons used per assay plate and 3) reducing the volume of water in which the cotyledons are extracted. Biddington and Thomas recommended incubating the cotyledons at 25°C for 18 hr. Recently Vallon et al. (1989) have shown a lag phase of 16 hr. between CK application and amaranthin production. These same authors showed that after a 20-hr. incubation, 56% more pigment was isolated than after a 16-hr. incubation. With this in mind, the incubation period should be extended

from 18 to at least 20 hr. Biddington and Thomas also recommended using ten cotyledons, but increasing the number used per assay will result in more pigment being synthesized in response to a given concentration of CK. Reducing the volume of water the cotyledons are extracted in will concentrate the pigment that is produced. Of these two options, the first one offers the greatest opportunity for improving sensitivity because there is a minimum volume of water needed to extract the cotyledons. In addition, the capacity of the cuvette used for taking optical density measurements may be a limiting factor.

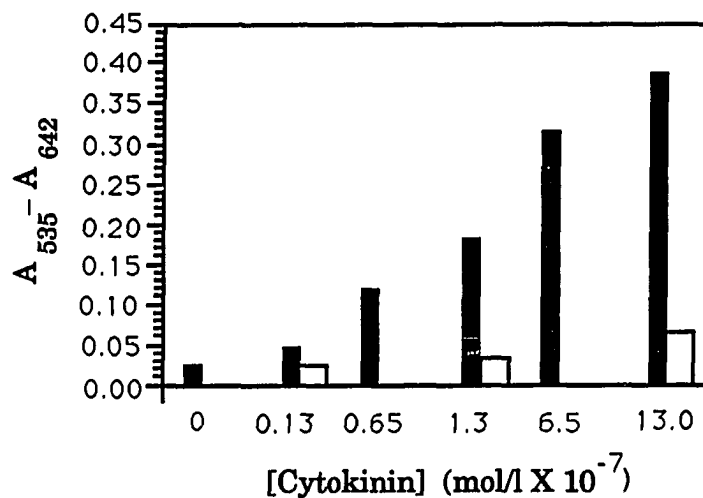


Figure 1. *Amaranthus* bioassay results for BAP and Z7G. The average responses at the indicated BAP concentrations (darkened bars), were derived from 10 assays (n=20). The average responses for the Z7G concentrations tested (1×10^{-8} , 1×10^{-7} and 1×10^{-6} M) were derived from a single assay (n=2) and plotted (open bars) next to their closest BAP equivalent

Literature Cited

- Biddington, N. L. and T. H. Thomas. 1973. A modified *Amaranthus* betacyanin bioassay for the rapid determination of cytokinins in plant extracts. *Planta* 111: 183-186.

- Horgan, R. 1984. Cytokinins. Pages 51-74 in M. B. Wilkins, ed. Advanced plant physiology. Longman Scientific and Technical, Harlow, Essex, England.
- LaMotte, C. E. 1990. Personal communication. Professor of Botany, Iowa State University, Ames, Iowa, 50011.
- Letham, D. S., L. M. S. Palni, G.-Q. Tao, B. I. Gollnow, and C. M. Bates. 1983. Regulators of cell division in plant tissues XXXIX. The activities of cytokinin glucosides and alanine conjugates in cytokinin bioassays. *Journal of Plant Growth Regulation* 2: 103-115.
- Vallon, U., J. A. Graf, and U. Kull. 1989. Influence of calcium and calcium and calmodulin antagonists on the cytokinin-induced amaranthin accumulation in *Amaranthus tricolor*. *Journal of Plant Growth Regulation* 8(2): 81-92.
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